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Response to Brosch et al.

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Abstract

We would like to respond to Brosch et al. regarding our manuscript "Expression of the Splicing Factor Gene *SFRS10* Is Reduced in Human Obesity and Contributes to Enhanced Lipogenesis" (Pihlajamäki et al., 2011b). Brosch performed RT-PCR in liver samples from 13 lean and 34 obese individuals, finding no differences in *SFRS10* or *LPIN1* expression. We wish to address points raised by Brosch, including experimental strategy and analysis of human *SFRS10* expression.

Experimental Strategy

Our overall goal was to identify genes or pathways altered in key insulin-responsive tissues in obese humans with or without type 2 diabetes (T2D). Liver biopsies were obtained during elective surgery from individuals who also had glucose tolerance testing (to define undiagnosed T2D). With this approach, we achieved an initial cohort of 13 metabolically

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SUPPLEMENTAL INFORMATION

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characterized individuals. Based on our prior observations (Patti et al., 2003), we recognized we would have limited power to detect single differentially expressed genes with this sample size. For this reason, and because no single gene is likely responsible for complex disorders such as obesity and T2D, we planned pathway-based analysis, as described in human T2D expression studies (Patti et al., 2003; Mootha et al., 2003). Our manuscript details pathway analysis, pattern validation in mice, hypothesis development, and selection of single genes for testing the hypothesis that splicing factors could contribute to metabolic phenotypes.

Single Gene Analysis

A human liver array study with 13 samples has limited power to identify single differentially expressed genes. Few, if any, would ever be expected to reach the Bonferroni-corrected p value suggested by Brosch ($p < 3.5 \times 10^{-6}$); this approach is not typically utilized for microarray analysis, due to the high number of comparisons (e.g., 14,500 probes). For single-gene analysis, we calculated q values (Bioconductor multitest function, Benjamini-Hochberg correction for multiple comparisons). As seen in Figure S1A, one *SFRS10* probe set demonstrated 37% reduction in expression in obese humans (200893_at, $q = 2.5\ 3\ 10^{-3}$), while another was 25% lower in obesity (200892_s_at, q = 0.09). A third probe set (210180_s_at) was not analyzed, as its signal was near background (lean, 42 ± 18 versus OB, 50 ± 17). We emphasize that *SFRS10* was not selected due to differential expression across the entire microarray, but due to its role as member of RNA processing gene set (n = 199) identified in pathway analysis.

Pathway Analysis

Pathway-based approaches (GenMAPP, GSEA) identified significant downregulation of genes of RNA processing and splicing in obese subjects (e.g., GenMAPP, Z7.5 RNA splicing, adjusted $p < 0.001$; GSEA, RNA processing set enriched, FDR 0.039) and correlation of this ontology to lipid accumulation. Further support came from parallel independent observations of downregulation of RNA processing genes in muscle from a Finnish population (GenMAPP Z11.5 RNA splicing, adjusted $p < 0.001$). Our collaboration was initiated when we viewed each other's data at an international meeting; these concordant patterns in two human populations led to our studies.

Rationale for Further SFRS10 Analysis

These independent human observations and similar patterns in murine obesity suggested a potential link between RNA processing and obesity-related metabolism (as shown in Figure 1 of Pihlajamäki et al., 2011b). Although there were several genes of interest within the overlapping set of RNA processing genes, *SFRS10* was a candidate of great interest as: (1) expression was downregulated in liver and muscle in obese humans and mice (Figures 1B and 1C [Pihlajamäki et al., 2011b]), (2) expression in liver significantly correlated with BMI ($r = -0.73$, Table S3 [Pihlajamäki et al., 2011b]), (3) protein expression was significantly decreased in obese mouse liver (Figure 1D [Pihlajamäki et al., 2011b]), (4) expression was altered by chronic insulin in HepG2 cells (Figure 1 [Pihlajamäki et al., 2011b]), and (5) siRNA targeting SFRS10 increased lipogenesis, expression of lipogenic genes, and lipid content versus scrambled siRNA-treated cells (Figure 2 [Pihlajamäki et al., 2011b]). By

contrast, knockdown of a constitutive splicing factor (SF3A1) did not alter triglyceride content or lipogenic expression (Figures S2A and S2B [Pihlajamäki et al., 2011b]). Guided by *Cell Metabolism* peer reviewers, we tested the role of SFRS10 in vivo. These studies demonstrated that SFRS10 heterozygous mice had upregulation of multiple lipogenic genes (concordant with cellular knockdown) and increased plasma VLDL (Figure 3 [Pihlajamäki et al., 2011b]), supporting a physiological role for SFRS10. Thus, several independent observations in humans, cells, and mice validated the selection of *SFRS10* as candidate for further studies.

Analysis of SFRS10 Expression by PCR

Brosch and colleagues analyzed a cohort of 47 human liver samples using PCR. While substantial inter-individual variation, as expected for human populations, could limit power to detect differential expression of *SFRS10*, we believe that several distinct mechanisms are likely to be responsible for differences in results:

Patient Population and Biopsy Approach

Our cohort compared a lean group (mean BMI 24) with an extremely obese group (mean BMI 53), of whom four had T2D diagnosed during study. The number of subjects with T2D in the Brosch cohort is not provided. Given that chronic insulin exposure reduced expression of SFRS10 in cells (Figure 1 [Pihlajamäki et al., 2011b]), it is possible that substantially increased fasting insulin levels in obese subjects (mean 148 pM, Table 1 [Pihlajamäki et al., 2011b]) might contribute to reduced SFRS10 expression, as suggested by the significant correlation between fasting insulin and liver SFRS10 expression ($r = -0.54$, Table S4 [Pihlajamäki et al., 2011b]). Fasting insulin also correlated with expression of six other RNA processing genes (Table S4 [Pihlajamäki et al., 2011b]). Together, these data suggest that more marked elevations in circulating insulin, associated with severe obesity, insulin resistance, and T2D, may have contributed to differences between study populations.

Our biopsies were taken in the fasting state during elective abdominal surgery, with general anesthesia, whereas Brosch obtained biopsies both during surgery and via percutaneous approaches. Were all performed in the fasting state? Were lean or postbypass individuals more likely to have had percutaneous, and obese subjects surgical, biopsies? If so, expression data may not be directly comparable across BMI ranges.

PCR Quantification of SFRS10 Variants

PCR analysis of *SFRS10* mRNA expression is complex. *SFRS10* has multiple alternatively spliced mRNA variants (17 in current Ensembl browser), which play an important role in the known autoregulation of SFRS10 protein (Stoilov et al., 2004). When present at high levels, SFRS10 binds its own exon 2, activating its inclusion and generating the Tra2b4 transcript, which is not translated due to a stop codon. Conversely, if SFRS10 protein is low, exon 2 is skipped, favoring the functional Tra2b1 isoform (Figure S4 [Pihlajamäki et al., 2011b]).

Brosch tested expression of *SFRS10* using a 5′ (forward) primer spanning exons 1–3 and 3′ (reverse) primer in exon 4 (Figure S1B). This leads to one PCR product, for Tra2b1 (Figure 1A in Brosch et al., 2012), but would not detect Tra2b4. By contrast, the microarray

quantified total Tra2b or all isoforms containing the common 3′ UTR, including both Tra2b1 and Tra2b4. Due to autoregulation, these differences in primer location are probably crucial. For example, the magnitudes of decreases in untranslated Tra2b4 are greater than for Tra2b1 in both SFRS10 heterozygous mice and SFRS10 knockdown cells (Figure S4B of Pihlajamäki et al., 2011b and Figures S1C and S1D). Therefore, it is possible that differences in relative expression of these variants underlie differences between our findings. Differences in relative abundance of SFRS10 isoforms may also alter splicing of downstream targets, including *Lpin1*. RNA sequencing may be helpful to directly address these possibilities.

We have now performed additional PCR-capillary electrophoresis to detect proportions of Tra2b1 and Tra2b4 with primers in exons 1 and 3 spanning alternatively spliced exon 2 (Figure S1B). Liver samples from 73 individuals (age 45.8 ± 7.8, BMI 45.1 ± 6.1, 24 male/49 female, described in Pihlajamäki et al., 2011a) were used. Although this data set does not include lean individuals, the proportion of Tra2b1 (without exon 2) correlated inversely with BMI ($r = -0.249$, $p = 0.035$) (Figure S1E). RT-PCR using primers in exons 7 and 8 did not demonstrate similar association. This provides additional validation of the association between *SFRS10* variants and obesity and highlights the complex regulation of *SFRS10* variants that potentially contributes to differences between our studies.

Additional Comments

Brosch and colleagues note that “only 2 of the 13 genes showed a similar change of expression [in mice] as seen in humans.” This is not correct. We tested expression of 5 genes (identified from human array analysis) in obese mice and found significantly reduced expression of 5 of 5 in liver and 4 of 5 in muscle (Figure 1C of Pihlajamäki et al., 2011b).

We also wish to clarify that additional samples used for LPIN1β/α ratio determination were obtained from 6 lean and 14 obese subjects of a cohort with similar metabolic profile described in a previous manuscript (Table S1 [Pihlajamäki et al., 2009]).

Together, we appreciate the work of Brosch to examine SFRS10 expression in an additional cohort. Differences in patient population, methods to detect *SFRS10* mRNA variants, and complicated autoregulation of *SFRS10* are all likely contributors to differences between our findings. We fully acknowledge that experimental analysis in cells and mice does not provide direct evidence for significance in humans and look forward to future studies further addressing the role of *SFRS10* in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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