**The Acromegaly Gene Expression Signature in Human Adipose Tissue Reveals Possible New Mechanisms for Enhanced Lipolysis and Insulin Resistance**

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**ABBREVIATED TITLE:** Analysis of Acromegalic Adipose Tissue

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**Structured Abstract:**

Context: Excessive GH production in acromegaly leads to multiple metabolic alterations, including insulin resistance and enhanced lipolysis.

Objective: to understand transcriptional changes which contribute to the metabolic effects of GH

Design. Prospective observational study from March 2011 to June 2012.

Setting: Tertiary Referral center at the University of Michigan Pituitary clinic

Participants: Patients with acromegaly or non functioning pituitary adenoma .

Intervention: The patients underwent clinical and metabolic. Explants of adipose tissue were assayed ex-vivo for lipolysis. Adipose tissue was analysed by RNAseq.

Main Outcome: differences in adipose tissue mRNA expression between acromegaly patients and controls.

Results: There was a trend for enhanced adipose tissue lipolysis in acromegaly patients (p=0.09).When correcting for BMI there was a significant decrease in insulin sensitivity in the acromegaly patients (P=0.0020). We observed several expected transcriptional changes (*IGF1*, *IGFBP3*, *SOCS2*, *CISH*) as well as several novel transcriptional changes, some of which may be important for GH signal regulation (*PTPN3* and *PTPN4*) and the effect of GH on growth and proliferation. Several transcripts could potentially be important in GH-induced metabolic changes. Induction of *TCF7L2*, *GFPT1* and *SCD* could contribute to insulin resistance, while induction of *LPL*, *NRIP1*, *ABHD5*, *ADRB3*, *TSHR*, *ACVR1C* could contribute to enhanced lipolysis. Expression of *HSD11B1* was reduced and GR was increased, predicting enhanced glucocorticoid activity, most likely contributing to metabolic effects.

Conclusions: We identified the acromegaly gene expression signature in human adipose tissue. The significance of altered expression of specific transcripts will enhance our understanding of the metabolic changes associated with acromegaly

**Introduction**

Acromegaly, excess growth hormone (GH) production secondary to a pituitary adenoma, has important metabolic effects. Insulin resistance, presenting as diabetes or impaired glucose tolerance, is found in most patients (1), and contributes to the enhanced morbidity (2). The two most significant effects of GH on metabolism in adipose tissue are insulin resistance and lipolysis (3). To study the effect of excess GH on adipose tissue, we used next generation RNA sequencing to study adipose tissue from acromegaly patients and controls. We found a distinctive pattern of changes in many transcripts that are highly associated with acromegaly. Many of these alterations are expected to contribute to the metabolic effect of GH and reveal novel mechanisms of GH-induced insulin resistance and lipolysis in adipose tissue.

**Methods**

**Patient recruitment**

The study was approved by the institutional review board of the University of Michigan Medical System. Written informed consent was obtained from all patients. Patients were recruited consecutively from those undergoing transsphenoidal adenomectomy at the University of Michigan for acromegaly or non-functioning pituitary adenoma over a 12 month period. Exclusion criteria were age <18, current hormone treatment including glucocorticoids, malignancy, inflammatory disease, diabetes type 1 and established pituitary hormone deficiencies. For each patient, a data sheet was completed including, age, sex, anthropometric measurements, diagnosis of hypertension, diabetes, results of blood tests and medications. Fasting blood samples were assayed for glucose (Siemens Advia 1800) and insulin (Life Technologies) as instructed by the manufacturers.

**Subcutaneous fat biopsy**

During the course of pituitary surgery a routine subcutaneous fat graft is utilized to seal the surgical field upon completion of the procedure. 500 mg of this fat graft was used for the study. ~100 mg were utilized for ex vivo lipolysis assay ~200 mg was snap frozen in liquid nitrogen and stored at -80 degrees for RNA preparation.

***Ex vivo* lipolysis**

25 mg pieces of adipose tissue were pre-incubated for 15 minutes in KRBH buffer (sigma) at 37°C and then incubated for 1 hour at 37°C in 300 μl KRBH in the presence or absence of isoproterenol 30nM in duplicate. Glycerol was assayed in supernatants using a glycerol assay kit (sigma) as instructed by manufacturer.

**Transcriptomic Analysis**

Total RNA was extracted from adipose tissue using the RNEasy kit (Qiagen)and its quality was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies). At the University of Michigan DNA Sequencing Core, cDNA libraries from polyA mRNA was prepared using TruSeq cDNA synthesis kit and sequenced using a HiSeq 2000 (Illumina). Samples were run on 2 lanes of a HiSeq 2000 (Illumina) generating 8 612 682 to 16 469 501 single-ended 50bp reads per sample. These were aligned to the human genome (Enembl GRCh37.69, Genbank Assembly ID GCA\_000001405.9) using TopHat version 2.0.6 (4), Bowtie version 2.0.2 (5) and Samtools version 0.1.18 . The transcripts with the lowest 40% quantile of average expression were removed from this analysis, for a total of 123292 total transcripts tested. Gene expression was analyzed using DESeq version 1.12.0 (6). All p-values were adjusted by the method of Benjamini-Hochberg (7). These subjects corresponded to the patients described in Table 1, with the exception of subjects 32 and 35 (both acromegaly patients), which had clinical data but no RNAseq data.

For re-analysis of the Huo dataset (8), we downloaded these data from the Gene Expression Omnibus (GSE2120) and compared the 48h treated control data to the 48h GH treated data. Analysis was performed using limma (version 3.16.7; (9)), with all p-values adjusted by the method of Benjamini-Hochberg (7)

**Statistics**

Statistical significance in this study was defined as a p-values of less than 0.05. All statistical tests were performed using the R package (version 3.0.1,(10)). Unless otherwise indicated, between group comparisons were analyzed using a Welch’s two sample t-test. All p-values were corrected for multiple observations by the method of Benjamini and Hochberg (7). To test for correlations we calculated Pearson's product-moment correlation. To test for gene set enrichment, either for gene ontology or KEGG categories, we used the GOseq package (version 1.12.0), taking sequence length bias into account and using the wallenius approximation for the null distribution (11). Resulting p-values were adjusted by the method of Benjamini and Hochberg (7). To test for enrichment of genes identified in the Huo *et al.* dataset (8), we used Fisher’s exact test.

To correct for the effects of BMI on insulin sensitivity, we generated a linear model using the natural logarithm of the HOMA-IR score as the dependent variable, and the BMI and the diagnosis as the independent variables. We observed no evidence of an interaction between BMI and the diagnosis in this model (p=0.753). This model had an adjusted R2 of 0.6241.

**Results**

**Patient characteristics**

Clinical and metabolic measurements were obtained for 9 acromegaly patients and 12 controls. Patient characteristics are shown in Table 1. There was no statistically significant difference in body mass index (BMI), abdominal circumference or weight. Acromegaly patients trended to be younger and taller than their controls.

**Acromegaly patients were more insulin resistant and had higher lipolysis**

Acromegaly patients trended to have higher fasting glucose levels (p=0.14) and higher fasted insulin (p=0.14, Figure 1A-B). When combined, we observed a trend towards higher HOMA-IR scores in the acromegalic patients (p=0.14, Figure 1C). We observed a significant association between BMI and insulin sensitivity (HOMA-IR score) across our subjects (p=0.014, R2=0.31), we therefore corrected for the wide range of BMI’s in this study and detected a significant decrease in insulin sensitivity in the acromegaly patients (a HOMA-IR score increase of 2.41, with a 95% confidence interval of 1.45-4.01; p= 0.0020; Figure 1D).

Subcutaneous adipose tissue chunks for lipolysis assay were available from 6 acromegaly patients and 9 controls. As shown in Figure 1C, acromegaly patients trended towards higher basal lipolysis (p=0.08), and higher lipolysis in the presence of isoproterenol (p=0.14). These data are consistent with previous reports linking GH signaling with increased lipolysis (12).

**Transcriptomic Analysis**

To determine which genes are altered in adipose tissue in acromegaly subjects, we performed a transcriptomic analysis of mRNA from subcutaneous adipose tissue from 7 acromegalic patients and 12 controls (Figure 2). We identified 728 transcripts from 199 genes that had significantly different expression in acromegaly, of these 43 genes were downregulated and 156 were upregulated in adipose tissue from the acromegalic patients. These transcripts form a signature identifying transcriptional differences in adipose tissue in response to long-term exposure to GH.

As shown in Figure 2, one acromegaly patient did not have this metabolic signature. This patient was the oldest in the cohort, had the weakest isoproterenol-induced lipolysis among the acromegaly patients, and also had the lowest blood glucose among all patients, including the controls.

Gene set enrichment analysis using GOseq (11) showed enrichment of genes in the gene ontology categories involved in development, signaling and lipid biosynthetic processes (Table 2). There was no significant enrichment of genes in molecular function gene ontology categories, or KEGG pathways.

Previous work by Huo *et al*. (8) examined an analogous *in vitro* system, where 3T3-F442A adipocytes were treated with GH for a variety of time points. The longest time point (48h) is potentially analogous to the chronic GH exposure that occurs in adipose tissue from acromegaly patients. We re-analyzed that data set, looking at only the 48h GH treatment time point. Out of the genes identified in our acromegaly analysis, 69 have mouse homologs, with 88 probes in that dataset. From examination of these probesets, we found 18 genes altered in the same direction in a statistically significant manner (or 19 %). Therefore the genes from the acromegaly dataset are significantly enriched in the GH treated dataset (p=1.95 × 10-7). The genes that reached statistical significance in both datasets were *Cish, Pld1, Fmo1, Phldb2, Igsf10, Ccng1, Wisp2, Ggct, Igfbp3, Igf1, Scd2, Scp2, Ptger3, Capn6, Socs2*.

**Established GH responsive genes are upregulated in adipose tissue from acromegaly patients**

Since acromegaly is caused by an overproduction of GH, we first analyzed known GH responsive genes. We found that GH responsive genes, including *IGF1* and *IGF-BP3*, are induced in acromegalic patients (Figure 3A-B). We found that growth hormone responsive genes, including *IGF1* and *IGF-BP3*, are induced in acromegalic patients (Figure 3A-B). IGF-1 has been shown to be induced in adipocytes exposed to GH (13), while there is no previous reports regarding IGF-BP3 induction. The confirmation of these previously reported acromegaly or GH dependent transcriptional changes strengthens our interpretation of other transcriptional changes.

Within the acromegalic subjects, there was a correlation between *IGF1* mRNA and levels of IGF-1 in serum in the acromegaly patients (R2=0.51, p=0.042; Figure 3C), reflecting an increased induction of *IGF1* in adipose tissue in acromegaly similar in its extent to serum IGF1 induction. Serum IGF1 is primarily thought to be derived from liver tissue (14). Our data demonstrates that adipose tissue may also be a source of IGF-1 under these conditions.

**Negative regulators of GH signaling are upregulated in acromegaly patients**

*SOCS2* AND *CISH*, both suppressors of cytokine signaling previously reported to be important in downregulating GH signaling, are upregulated in acromegaly (Figure 3D). These genes have been previously reported to be induced in liver and muscle by GH (15), while *SOCS2* has also been reported to be induced in adipocytes by GH (13). Induction of expression of these genes reflects activation of a negative feedback loop in response to chronic high GH (15), while *SOCS2* has also been reported to be induced in adipocytes by GH (13). These genes likely reflect the upregulation of a negative feedback loop in response to chronic high growth hormone levels.

We also observed induction of the tyrosine phosphatases, *PTPN3* (also called PTP-H1) and *PTPN4* in acromegaly. *PTPN3* has been reported to bind GH receptor *in vitro* in the presence of GH (16), and its overexpression reduces STAT5 signaling in response to GH (17). *Ptpn3* Knockout mice have excessive GH activity, as demonstrated by excessive growth accompanied by a strong induction liver *IGF1* mRNA and serum IGF-1 (17). The induction of *PTPN3* that we have observed in acromegaly suggests that this may be an additional negative feedback pathway induced to reduce GH signaling in acromegaly.

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**Genes controlling DNA replication, proliferation and apoptosis**

We observed difference in expression of several different genes regulating cellular proliferation in acromegalic subjects. Cyclin E, which is important for transition from G1 to S phase in the cell cycle, and Cyclin G, which is important for DNA repair, were increased in acromegalic patients, and cyclin dependent kinase inhibitor B was decreased. Nucleosome assembly protein 1-like 1 (*NAP1L1*) and *ORC2* (origin recognition complex, subunit 2), which are important for DNA replication and the antiapoptotic regulators *BAG1*(BCL2-associated athanogene 4) and calpain 6 were also induced . The effect of GH on these transcripts has not been reported before, and these could account for the effects of enhanced cell proliferation and repressed apoptosis in response to GH.

**Transcripts altered in acromegaly that may contribute to insulin resistance**

We observed no difference in expression of canonical transcripts important for insulin signaling and response to insulin in adipocytes, including insulin receptor (*INSR*), *IRS1, IRS2*, *AKT1-3*, or *SLC2A4* (or GLUT4; see Supplementary Figure 1A-G). This indicates that the observed insulin resistance is not caused by transcriptional changes in these genes. In fact, most of these genes trended to be more highly expressed in the adipose tissue from these insulin resistant patients, potentially underlying an upregulation that compensates for an alternative insulin resistant mechanism.

One previously identified candidate is the phosphoinositide-3-kinase, regulatory subunit 1 (*PIK3R1*, also called p85α), which was previously found to be induced by GH in adipose tissue (18) and thought to contribute to GH-induced insulin resistance (18). In our study it trended to be expressed higher in the acromegaly patients, but the difference was not statistically significant (Figure 4A).

We therefore examined for alternations in non-canonical insulin sensitivity pathways. We observed that glutamine-fructose-6-phosphate transaminase 1 (*GFPT1*), the rate-limiting enzyme of the hexosamine pathway, was induced in the acromegaly patients (Figure 4B). It has been established that higher expression of this enzyme is associated with insulin resistance, most likely through covalent glucosamine modification of enzymes of the insulin signaling pathway (19) and its up-regulation may contribute to insulin resistance in the acromegaly patients. Previous work has identified several glucosamine-modified proteins in the insulin signal transduction pathway (20,21).

We also observed an increase in *TCF7L2* (also called TCF4), a transcription factor regulating many metabolic genes that has been identified via genome wide association studies as associated with diabetes (22) , suggesting that its higher expression in acromegaly may contribute to insulin resistance.

Another possible link between acromegaly and insulin resistance may be the fatty acid desaturases Stearoyl-CoA desaturase (*SCD*, delta-9-desaturase) and fatty acid desturase 2 (*FADS2****)***, delta-6-desaturase). These enzymes function to add double bonds to acyl chains during lipogenesis and their activities have been associated with metabolic syndrome (23,24). Both of these genes were induced in acromegaly patients, possibly contributing to insulin resistance in these patients. Expression of both transcripts has recently been shown to be induced by GH in mice (25).

**Transcripts regulating lipid metabolism and localization that may contribute to enhanced lipolysis**

To determine the potential causes of the increased lipolysis observed in Figure 1E and F, we examined the expression of human lipases in these adipose tissues (26) and in adipose tissue biopsies from GH deficient patients after treatment with GH (27). Notably, other studies have found no change or even a reduction in LPL activity in response to GH treatment (28,29).

In addition to these lipase genes, two important direct regulators of hormone sensitive lipase and adipose triglyceride lipase activity in adipocytes, nuclear receptor interacting protein 1 (*NRIP1*, also called RIP140 see Figure 4D) (30), and abhydrolase domain containing 5 (*ABHD5*, also called CGI58, see Figure 4E) (31), are expressed in higher levels in acromegaly, and are strong candidates for the induction of lipolysis by GH. *NRIP1* is also a transcription regulator of genes involved in lipid and glucose metabolism (30) and its induction may also contribute to the effects observed in carbohydrate homeostasis.

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. We found that this gene is also upregulated in the acromegaly patients. Mice with liver specific knockout of *TCF7L2* are hypoglycemic, while transgenic mice overexpressing liver *Tcf7l2* are hyperglycemic. Expression of *TCF7L2* in subcutaneous fat is higher in insulin resistant subjects, and expression of several splice isoforms is reduced in subcutaneous fat and in liver following bariatric surgery We also observed an increase in *TCF7L2*, a transcription factor regulating many metabolic genes has been identified via genome wide association studies as associated with diabetes (22). We found that this gene is also upregulated in the acromegaly patients. Mice with liver specific knockout of *TCF7L2* are hypoglycemic, while transgenic mice overexpressing liver *Tcf7l2* are hyperglycemic. Expression of *TCF7L2* in subcutaneous fat is higher in insulin resistant subjects, and expression of several splice isoforms is reduced in subcutaneous fat and in liver following bariatric surgery (22) .

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**Transcripts regulating lipid metabolism and localization that may contribute to enhanced lipolysis**

. We observed no significant difference in expression of hormone sensitive lipase (*LIPE*), adipose triglyceride lipase (*PNPLA*) or monoglycerol lipase (*MGLL*; Figure 4C). Lipoprotein lipase (*LPL*), the lipase important for lipolysis of triglycerides in apolipoproteins, was significantly expressed more in acromegaly patients (figure). A strong induction of *LPL* expression in response to GH and absence of change in HSL was demonstrated before in a preadipocyte cell line To determine the potential causes of the increased lipolysis observed in Figure 1E and F, we examined the expression of human lipase in these adipose tissue lysates. We observed no significant difference in expression of hormone sensitive lipase (*LIPE*), adipose triglyceride lipase (*PNPLA*) or monoglycerol lipase (*MGLL*; Figure 4C). Lipoprotein lipase (*LPL*), the lipase important for lipolysis of triglycerides in apolipoproteins, was significantly expressed more in acromegaly patients (figure). A strong induction of LPL expression in response to GH and absence of change in HSL was demonstrated before in a preadipocyte cell line (26) and in adipose tissue biopsies from GH deficient patients after treatment with GH (27). Notably, other studies have found no change or even a reduction in LPL activity in response to GH treatment (28,29).

In addition to these lipase genes, two important direct regulators of hormone sensitive lipase and adipose triglyceride lipase activity in adipocytes, nuclear receptor interacting protein 1 (*NRIP1*, also called RIP140 see Figure 4D) (30), and abhydrolase domain containing 5 (*ABHD5*, also called CGI58, see Figure 4E) (31), are expressed in higher levels in acromegaly, and are strong candidates for the induction of lipolysis by GH. *NRIP1* is also a transcription regulator of genes involved in lipid and glucose metabolism (30) and its induction may also contribute to the effects observed in carbohydrate homeostasis.

We also examined the expression of three G-protein coupled receptors which induce lipolysis, the beta 3 adrenergic receptor (*ADRB3*), TSH receptor (*TSHR*) and Oxytocin receptor (*ACVR1C*), were induced very significantly in acromegaly patients compared to the controls, though at relatively low levels. *ADRB3* has previously been shown to be induced in mouse adipose tissue in response to long term GH treatment (32). The lipid transport proteins, sterol carrier protein 2 (*SCP2*. *APOL6* and *APO4*) were also induced in acromegaly patients.

Angiopoietin like 4 (*ANGPTL4*), which has been recently shown to be induced in muscle in response to acute GH treatment, and suggested as a possible regulator of lipolysis (33) but was not observed to be significantly induced in adipose tissue in the acromegaly patients in our study.

As far as glycogen synthesis is concerned, two glycogenolysis enzymes, muscle glycogen phosphorylase (*PYGM*) and phosphohexomutase (*PGM3*), were also induced in the acromegaly patients. The significance of these in adipose tissue, or changes in glycogen content in acromegalic adipose tissue has not been characterized.

**Glucocorticoid regulation**

11βHydroxysteroid dehydrogenase 1 (*HSD11B1*), the enzyme that inactivates cortisol, was reduced 4 fold in acromegaly patients (Figure 4F). The downregulation of expression and activity of this enzyme by GH/IGF1 has been confirmed both *in vitro* (34)(34), in GH deficient patients treated with GH (35)(35) and in acromegaly patients (36,37) .(36,37) . In addition, we found higher expression of the glucocorticoid receptor (*NR3C1*) in acromegaly patients. The net effect of these two changes is predicted to enhance glucocorticoid-responsive effects. Glucocorticoids have profound metabolic effects in adipose tissue (38) and the enhanced glucocorticoid effect may contribute to both insulin resistance and lipolysis in acromegalic patients.

**Summary**

In this study we have described a transcriptional signature in adipose tissue from subjects with acromegaly. We identified 157 adipose tissue transcripts altered in acromegaly. Some of these genes may be direct targets of increased GH or IGF-1 signaling in adipose tissue, whereas others may be secondary adaptations to this condition.

The main limitation of our study is the small sample size. The fact that 6 of the 7 patients consistently had a uniform change of expression of these genes and that despite rigorous correction for multiple observations the changes were still significant, suggests that we are able to draw valid conclusions about adipose tissue in acromegalic patients even from this small cohort. Furthermore, as mentioned throughout this paper, our data agrees with several previous studies in animal models and patients. The confirmation of these previously reported GH-dependent transcriptional changes strengthens our interpretation of other transcriptional changes.

Interestingly, one of the patients, who had some unique clinical features and did not display insulin resistance and enhanced lipolysis, also did not share the pattern of expression of the described genes with the rest of the acromegaly patients. These data suggest that the transcriptional signature identified in this study is tightly correlated with clinical phenotypes in acromegaly patients.

These data provide a variety of novel transcriptional changes that may be causative of the co-morbidities associated with acromegaly. Further studies in animals and cells using knockout or overexpression of specific transcripts may verify which of the changes is crucial in metabolic effects of GH in adipose tissue.

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**Table Legends:**

**Table 1: Clinical characteristics.** Data represents mean +/- standard error.

**Table 2:** **Gene set enrichment of transcripts from acromegalic white adipose tissue.** Gene ontology biological process categories enriched by in transcripts that differ between acromegaly and control subjects. DE Genes indicates the number of differentially expressed genes in this category, Total is the total number of genes in that category. There were no significantly enriched gene ontology molecular function categories or KEGG pathways..

**Figure Legends**

**Figure 1: Acromegalic patients tended to have reduced insulin sensitivity and higher lipolytic activity than their controls.** A) Fasting blood glucose levels. B) Fasting insulin levels. C) HOMA-IR score. D) Plot of each subject’s BMI compared with the natural logarithm of their HOMA-IR score. A best fit line was drawn for each subject pool. E) Unstimulated *ex vivo* lipolysis as measured by glycerol release from excised white adipose tissue from control or acromegaly patients. F) Glycerol release after stimulation with 30 nM isoproterenol. Data is presented as mean +/- standard error of the mean.

**Figure 2: Differential expression of genes in white adipose tissue from subject with compared to controls.** A) Heatmap of the differentially expressed genes in white adipose tissue. In where several transcripts derived from a single were identified as differentially expressed, only the most significant was shown. Individual values are colored as the log fold change for a particular gene in a particular subject compared to the average expression of that gene across all cohorts, with brown indicating less expression and green indicating more expression (designated in the key as Row Z-score). The bar across the top indicates the subject’s diagnosis, red for acromegaly and blue for controls.

**Figure 3: Previously reported GH targets are differentially expressed in acromegaly subjects.** A) mRNA Expression of A) *IGF1* and B) *IGFBP1* transcript levels in adipose tissue from control and acromegalic patients. Only the highest expressing transcript is shown for each gene. C) Comparason between *IGF1* mRNA and IGF-1 serum levels in patients with acromegaly. mRNA Expression of *CISH* and *SOCS2* in adipose tissue from acromegaly patients and controls. Asterisks indicate p<0.05. Barplots are presented as mean +/- standard error of the mean.

**Figure 4: Expression changes of selected genes, potentially responsible for lipolytic or insulin sensitivity alterations in acromegaly patients.** mRNA Expression profile of genes potentially involved in insulin sensitivity (A-B) or lipid metabolism (C-F). C) Lipases in human tissue were examined, only lipases with >100 FPKM are shown. Asterisks indicate p<0.05. Data indicates mean +/- standard error of the mean.

**Supplementary Data**

**Supplementary Table 1: Expression changes between control and acromegaly subjects.** Calculated expression, and expression changes for each transcript are shown along with raw, and adjusted p-values and the fold change for acromegaly data.

**Supplementary Figure 1: Expression changes of selected insulin signal transduction genes.** mRNA Expression profile of genes involved in insulin signaling. Data indicates mean +/- standard error of the mean.

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