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Differential gene signature in adipose tissue depots of growth hormone transgenic mice

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Abstract

Bovine growth hormone transgenic (bGH) mice mimic the clinical condition of acromegaly, having high circulating growth hormone (GH) levels. These mice are giant, have decreased adipose tissue (AT) mass, impaired glucose metabolism, and shortened lifespan. The detrimental effects of excess GH have been suggested, in part, to be due to its depot-specific actions on AT. To investigate this relationship, we evaluated the gene expression, biological mechanisms, cellular pathways, and predicted miRNAs in two AT depots (subcutaneous [Subq] and epididymal [Epi]) from bGH and littermate controls using RNA sequencing (RNAseq) analysis. Two analyses on the differentially expressed genes (DEG) were performed: 1) comparison of the same AT depot between bGH and WT mice (genotype comparison) and 2) comparison of Subq and Epi AT depots within the same genotype (depot comparison). For the genotype comparison, we found a higher number of significant DEG in the Subq AT depot of bGH mice compared to WT controls, corroborating previous reports that GH has a greater impact on the Subq depot. Furthermore, most of the DEG in bGH mice were not shared by WT mice, suggesting that excess GH induces the expression of genes not commonly present in AT. Through gene ontology and pathway analysis, the genotype comparison revealed that the DEG of the Subq depot of bGH mice relate to fatty acid oxidation, branched chain amino acid degradation, and the immune system. Additionally, the AT depot comparison showed that the immune cell activation and T cell response appear upregulated in the Subq compared to the Epi AT depot. The miRNA prediction also suggested a modulation of T cell-related biological process in Subq. In summary, the present study provides a unique resource for understanding the specific differences in gene expression that are driven by both excess GH action and AT depot location.

Keywords

Growth hormone; adipose tissue; RNAseq; bGH mice; depot differences; immune response

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1. Introduction

Acromegaly is an acquired condition characterized by increased circulating growth hormone (GH) and insulin-like growth factor-1 (IGF-1) levels and profound metabolic complications that ultimately lead to premature death (1). The giant bovine GH transgenic (bGH) mice share many features with the clinical condition of acromegaly and have been extensively studied to evaluate the effects of excess GH action *in vivo* (2–5). Similar to acromegaly, bGH mice have elevated serum GH and IGF-1 levels (4, 5), but individuals with acromegaly exhibit high basal, yet pulsatile GH serum levels (6, 7), while bGH mice have a life-long, continuous elevation in GH. GH has an anabolic effect on muscle and a catabolic effect on adipose tissue (AT) (2). As such, bGH mice and acromegaly are associated with an intriguing ‘lean, but unhealthy, phenotype’ and can be regarded as a non-fatty liver model of lipodystrophy. That is, adult bGH mice exhibit insulin resistance and hyperinsulinemia, even though body composition is favorable with dramatically reduced fat mass and increased lean mass (2, 3, 7). Patients with acromegaly and bGH mice both exhibit increased incidence of cancer (8), cardiovascular (9–11) and kidney diseases (12–14), and significantly decreased lifespan when compared to controls with normal GH action (3, 15, 16).

AT, a highly dynamic organ, has drawn attention in the quest to understand how GH impacts health and longevity. GH is known to decrease lipid accumulation in AT (17) by promoting lipolysis and preventing lipogenesis (18, 19). However, GH’s impact on AT extends beyond its role in the lipogenic/lipolytic processes (20). GH also influences preadipocyte differentiation and proliferation (21), preadipocyte senescence (22), fibrosis (23, 24), beiging (2), adipokine secretion (20), and immune cell abundance (25). Furthermore, *in vivo*, GH has been shown to influence AT in a depot-specific manner, as subcutaneous (Subq) and intraabdominal AT tissues respond differently and with varying intensities to GH action (20). Interestingly, the depot most impacted by GH seems to vary by species. In humans, the visceral depot has been suggested to be the most affected by alterations in GH action, and the greatest reduction of AT mass occurs in the visceral depot in patients with active acromegaly (26) and in GH deficient patients treated with GH (27). In mice, where more invasive techniques can be employed, the Subq depot is most altered with dramatic changes in AT mass compared to other depots (28). As for bGH mice, AT mass is reduced, which becomes more prominent with advancing age and in male mice (7). bGH mice also have 1) altered adipokine production with reduced leptin and adiponectin (29–31), 2) altered hormone sensitivity with reduced IGF-1 receptor (IGF-1R) and GHR expression levels (32), 3) increased extracellular matrix deposition (most prominently in Subq) (24), 4) increased beiging markers in Subq depots (33), 5) impaired preadipocyte differentiation of mesenchymal stem cells in Subq (25), 6) increased senescent cell burden of preadipocytes in all depots except perigonadal (epididymal) (22), and 7) AT immune cell infiltration with greater macrophage and regulatory T cell infiltration of only Subq depots (25).

Even though GH is well known to impact AT metabolism and plasticity, the molecular mechanisms and pathways responsible for GH’s depot-specific effects are still largely unknown. Thus, this study, using RNA-sequencing (RNAseq), explored the molecular mechanisms and cellular pathways that are altered in a depot-specific manner in six month

old male bGH and wild type (WT) mice. Two white AT depots were studied: Subq and epididymal-intraabdominal (Epi). These two depots were chosen as they have routinely shown the most dramatic differences in mass and phenotype in our bGH mouse studies with GH and AT. Two broad analyses were performed in this study. The first analysis compared the same AT depot between bGH and WT mice (genotype comparison). The second analysis compared two different AT depots - Subq and Epi AT depots - within the same genotype (AT depot comparison). Here, we present the gene expression profiles, biological processes, molecular mechanisms, and cellular pathways that may contribute to the AT depot-specific differences and responses to GH excess.

2. Materials and Methods

2.1 Animals

The bGH mice, derived and maintained in a C57BL/6J background, were bred and maintained at Ohio University. The bGH mice were generated using a metallothionein transcription regulatory element for the bGH complementary DNA as previously described (34). Mouse genotypes were determined by polymerase chain reaction (PCR) using tail samples. Three bGH and three WT male mice at six months of age were used. Mice were fed with a standard laboratory rodent chow (ProLab RMH 3000), provided food and water *ad libitum*, and were housed in a 25°C room with controlled light cycles of 14 hours light/10 hours dark. All procedures were approved by the Ohio University Institutional Animal Care and Use Committee.

2.2 Adipose Tissue Depots

Two white AT depots were studied: inguinal-subcutaneous (Subq) and epididymal-intraabdominal (Epi). Mice were euthanized by cervical dislocation after being rendered unconscious by CO₂, and AT samples were dissected and weighed. For RNA isolation, tissue was flash frozen in liquid nitrogen and stored at -80 °C until further processing.

2.3 Total RNA and Messenger RNA (mRNA) Isolation

Total RNA was isolated from AT depots with Trizol Reagent from Invitrogen, following the manufacturer's protocol with minor changes. Briefly, 1 ml of Trizol reagent was homogenized with 80–100 mg of frozen AT. Samples were then centrifuged at $17,000 \times g$ for 5 minutes at 4°C. Using a syringe, most of the lipid layer located on the top of the solution was removed without disturbing the Trizol. Next, 200 µL of chloroform was added to the sample and mixed, followed by centrifugation at $\sim 17,000 \times g$ for 15 minutes at 4 °C. The clear aqueous phase was transferred to a new tube, and 0.5 mL of 100% isopropanol was then added to the aqueous phase. Samples were centrifuged at $12,000 \times g$ for 10 minutes at 4 °C, and then the supernatant was removed leaving only the RNA pellet. The RNA pellet was washed three times as follows: 1 mL of ice cold 75% ethanol was added to the RNA pellet and mixed, then the sample was centrifuged at $17,000 \times g$ for 15 minutes at 4 °C, the pellet was air-dried, and resuspended using 50 µL of RNAase free water.

The quantity and quality of total RNA were measured with the NanoDrop ND-2000c (Thermo Scientific). The RNA used for subsequent experiments had a 260/280 and a

260/230 ratio 1.8 to ensure purity and quality of the RNA. Subsequently, mRNA was isolated from the total RNA samples with the Dynabeads mRNA Purification Kit (Invitrogen) according to the manufacturer's protocol. The quantity and quality of the isolated mRNA were determined by the Agilent 2100 Bioanalyzer (Agilent Technologies) at the Ohio University Genomics Facility. The mRNA used for RNA-seq had an RNA Integrity Number 8.

2.4 cDNA Library Preparation and RNA Sequencing

Subq and Epi mRNA samples isolated from three bGH mice and three control mice were used to create twelve cDNA libraries (1 mouse per library and 3 libraries per group). The cDNA libraries were produced using the Ion Total RNA-Seq Kit (Life Technologies) following the manufacturer's protocol. Briefly, 20 ng of mRNA was fragmented and ligated to identifier adaptors. Reverse transcription was performed to obtain cDNA, which in turn, was amplified through polymerase chain reaction to obtain cDNA libraries. The cDNA libraries were then sequenced using a single end methodology in an Ion Torrent Personal Genome Machine at the Genomics Facility (Ohio University).

2.5 RNA-Sequencing (RNA-seq) Analysis

RNA-seq analysis was conducted using the mm10 (*Mus musculus*) reference genome sequence version. This version was released in July 2012 by the Genome Reference Consortium (Ensembl, 2012). Using the online tool Galaxy (<https://usegalaxy.org/>), adapters were removed, and reads were trimmed for quality with Trimmomatic v0.3, using default settings. Reads were aligned using the hierarchical indexing for spliced alignment of transcripts (HISAT) v2.0.4 program. Independently, gene expression was calculated by running Cufflinks version 2.1.0 on the alignments from HISAT. Only reads with a Phred score 20 (which corresponds to 99% accuracy) were used in the analysis; p-values with false discovery rate (FDR) correction were used to determine statistical significance. The Program R (version 3.5.1) through vegan and ggfortify libraries was used to create a principal component analysis (PCA) to examine the spatial differentiation of gene expression between genotypes and AT depots. Genes were initially filtered by subgroup (i.e. genotype and adipose tissue, such as bGH epi), and any subgroup that contained two or more non-zero values were kept in the dataset. Two standardized and centered PCAs were performed on the same dataset, clustered by AT type and genotype separately.

2.6 Statistical Analysis

Gene expression is given in Fragments Per Kilobase of transcript per Million mapped reads [FPKM] units. Cuffdiff first sums the FPKM for transcripts sharing the same gene. The log (fold_change) was calculated by dividing the FPKM distributions of condition 1 by that of condition 2 and then applied log to the division result. A cutoff of 1.5 fold change was used for analysis with FDR < 0.05 considered statistically significant.

2.7 Gene ontology (GO) and pathway analysis of DEGs

The gene ontology (GO; <http://geneontology.org/>) enrichment of DEGs was implemented by the hypergeometric test, in which p-value is calculated and adjusted as a q-value. GO terms

with $q < 0.05$ were considered significantly enriched. Ipathway software from Advaitabio (<https://ipathwayguide.advaitabio.com>) using the KEGG database (Kyoto Encyclopedia of Genes and Genomes) was implemented to identify the most significantly changed pathways. This software uses the impact analysis method in which pathways are identified taking into account over-representation of DEG in a given pathway and the gene expression measurement as well as the directional interaction of the genes within the pathway. The underlying pathway topologies were obtained from the KEGG database. A hypergeometric test with FDR correction was used; $q < 0.05$ was considered significantly enriched. For miRNA prediction, the Ipathway software uses the databases MicroCosm Targets version 5 and Targetscan version: Mouse: 7.1.

3. Results

3.1 Excess GH has a greater impact on the number of significant DEG in the Subq AT depot than in the Epi depot

RNAseq was used to identify differentially expressed genes (DEG) in two AT depots (Subq and Epi) between bGH and WT mice. Two broad contrasts were performed using these samples: 1) genotype comparison (WT Subq vs. bGH Subq; WT Epi vs. bGH Epi) and 2) AT depot comparison (WT Subq vs. WT Epi; bGH Subq vs. bGH Epi). The objective of the genotype contrast was to evaluate the effect of excess GH in distinct AT depots. On the other hand, the main aim of the AT depot contrast was to evaluate the differences in DEG between AT depots within a given genotype (i.e. excess GH or normal GH action) (Table 1).

The genotype comparison (Figs 1A and 1C) had fewer DEG than the AT depot comparison (Figs 1B and 1D). In the genotype comparison (WT vs. bGH), 822 genes were significantly changed when comparing the Subq depots, and 175 genes were differentially expressed when contrasting the Epi depots. The AT depot comparison (Epi vs Subq) showed 527 significantly altered genes when comparing depots of WT mice, and 1542 differentially altered genes in the AT depot comparison of bGH mice (Table 1). The three-fold increase in genes altered in bGH mice suggests that excess GH exacerbates depot differences in gene expression and thus, results in greater depot distinction than is apparent in WT mice. Principal component analysis (PCA) showed a greater variation in the gene expression when comparing AT depots (Epi vs. Subq) than when comparing between genotypes (WT vs. bGH) (Figs 1E and 1F). Furthermore, contrasts between bGH and WT mice (genotype comparison) revealed that the Subq AT depot had more significantly altered genes than the Epi AT depot (Figure 1C). Interestingly, based on the number of DEG, GH impacts gene upregulation and downregulation nearly equally.

3.2 Excess GH exacerbates gene expression differences between AT depots, in part, by inducing the expression of genes not usually detected in the AT of WT mice

To better understand the genotype and depot differences seen in gene expression, the DEG were separated into downregulated and upregulated genes. The 20 most downregulated and upregulated genes according to their significance (FDR) are shown in Fig. 2, and a full list with their respective gene expression level (FPKM) and FDR is shown in suppl. tables 1–8.

As shown in the DEG gene lists and both genotype (Fig 2C) and depot (Fig 2G) comparisons, many genes are expressed in one AT depot while absent in the other.

To understand the differences and similarities between the comparisons, the DEG lists of the genotype and depot comparisons were analyzed, establishing which genes are uniquely expressed, shared, or divergent (Figures 3 and 4). For the genotype comparison, the gene lists of the downregulated (suppl. 1 and 2) or upregulated genes (suppl. 3 and 4) found in the Subq and Epi AT depots of bGH mice compared to controls were used to perform this analysis. The bGH mice had 472 and 113 downregulated genes in Subq and Epi AT depots, respectively (Figure 3B, suppl. tables 1 and 2) while 350 and 62 genes were upregulated in the bGH Subq and Epi AT depots, respectively, compared to controls (Figure 3D, suppl. tables 3 and 4). Furthermore, 20 downregulated genes (3.5% of the total downregulated genes) (Figs. 3A and 3B) and 12 upregulated genes (3% of the total upregulated genes) (Figs. 3C and 3D) were shared between the Epi and Subq AT depots of bGH mice. Interestingly, several genes were regulated in opposite directions. Specifically, four genes were downregulated in Subq and upregulated in Epi of bGH mice, and another four genes were upregulated in Subq but downregulated in the Epi of bGH mice when compared to WT mice (Fig. 3E).

For the AT depot comparison, the WT and bGH mice had 294 and 907 downregulated genes, respectively, in the Subq AT depot compared to Epi (Figure 4B, suppl. tables 5 and 6). Likewise, 233 genes in WT mice and 635 genes in bGH mice were upregulated in the Subq AT depot compared to Epi AT depot (Figure 4D, suppl. tables 7 and 8). Lists of the downregulated or upregulated genes found in the Subq AT compared to the Epi AT depot of WT or bGH mice were used to establish the genes that are uniquely expressed, shared, or divergent in the AT depot comparison (Figure 4). We identified that 129 downregulated genes (12% of the total downregulated genes) (Fig. 4B) and 171 upregulated genes (25.5% of the total upregulated genes) in the AT depot comparison were shared between WT and bGH mice (Fig. 4D). The 20 most downregulated (out of the 129 downregulated genes mentioned above) and upregulated shared genes (out of the 171 upregulated genes mentioned above) of the depot comparison of bGH mice are shown in Figure 4A and 4C. Nine genes had opposite trends in Subq AT vs. Epi AT in bGH or WT mice as shown in Fig. 4E.

Interestingly, both the genotype and the AT depot comparison showed that the percentage of shared DEG was low compared to the percentage of unique DEG, indicating that gene expression varied in a depot specific manner and as a function of GH. That is, for the genotype comparison, 452 downregulated genes (80% of the total downregulated) and 338 upregulated genes (84.5% of the total upregulated) were uniquely expressed differentially in the Subq AT depot of bGH mice compared to controls. Similarly, 93 downregulated genes (16.5% of the total downregulated) and 50 upregulated genes (12.5% of the total upregulated) were uniquely expressed in the Epi AT depot of bGH mice compared to controls (Figs. 3B and 3D). Furthermore, the AT depot contrast shows that WT mice had 165 uniquely downregulated genes (15.4% of the total downregulated) and 62 uniquely upregulated genes (8.9% of the total upregulated) in the Subq AT compared to the Epi AT depot. In comparison, bGH mice have more uniquely DEG in the Subq AT with respect to

the Epi AT depot with 778 uniquely downregulated genes (72.6% of the total downregulated) and 464 uniquely upregulated genes (66.6% of the total upregulated) (Figs. 4B and 4D). These results suggest that excess GH exacerbates the amount of DEG in the AT by inducing the expression of genes that are atypical of the AT in WT mice.

3.3 Gene ontology (GO) analysis: Upregulated genes in the Subq AT depot of both WT and bGH mice showed altered GO terms related with immune response and activation

To better understand the biological and functional significance of the DEG, we further analyzed either the downregulated or upregulated genes of both the genotype and AT depot comparisons with a gene ontology (GO) enrichment analysis tool. Upregulated and downregulated genes were classified into three GO categories: 1) biological process, 2) molecular function, and 3) cellular component. The most significant terms for each of the categories are summarized in Figure 5 for genotype comparisons and Figure 6 for the AT depot comparisons.

Within the Subq AT depot, the downregulated genes in bGH mice vs. WT mice had the most significant alteration of biological processes related to ‘fatty acid oxidation,’ ‘branched-chain amino acid metabolic process,’ and ‘brown fat cell differentiation’ (Figure 5A, yellow bars). Ion binding and oxidoreductase activity were the most significantly altered molecular functions (Figure 5A, red bars), and ‘cytoplasmic part’ and ‘mitochondrial part’ were the most altered cellular components (Fig. 5A, blue bars). For the upregulated genes, the five most significantly altered biological processes were all related to immune system biology. Likewise, the ‘cellular component’ and ‘molecular processes’ categories showed ‘T cell receptor binding’ and ‘T cell receptor complex’ as some of the most significant terms in those categories (Fig. 5B). In striking contrast to the results showed for the Subq AT comparison (Fig. 5A and B), genotype comparison in the Epi had very few significant differences in the three categories examined in GO (Fig. 5C and 5D).

For the depot comparison in WT mice, the downregulated genes (in Subq vs Epi) showed that while the most significantly altered biological processes were related to cell movement (Figure 6A, yellow bars), the most significant terms in the cellular component category included ‘extracellular matrix’ and ‘collagen-containing extracellular matrix’ (Fig. 6A, blue bars). On the other hand, the GO analysis of downregulated genes in the Subq AT depot vs. Epi AT of bGH mice showed that the most significant biological processes included ‘organic acid metabolic processes’ and ‘oxidation reduction processes’ (Fig. 6C, yellow bars). Interestingly, similar to the genotype comparison, the upregulated genes in the depot comparison (Subq vs Epi) in both genotypes revealed that the five most significantly altered biological processes were related with immune responses and processes, including ‘leukocyte activation’ and ‘lymphocyte activation’ (Fig. 6B and 6D, yellow bars). Moreover, the significant terms of the molecular function category were related to protein or molecule binding (Figs. 6B and 6D, red bars), such as the ‘MHC class II protein complex binding.’

3.4 DEG of the genotype and depot comparisons are related to biological pathways involving fatty acid and amino acid metabolism as well as immune cell regulation

To further integrate and analyze the upregulated and downregulated genes, the DEG of both genotype and AT depot pairwise comparisons were used to perform pathway analysis. Of note, the pathway analyses combined the upregulated and downregulated DEG, unlike the GO analyses described above. The most significantly changed pathways showed some similar trends to the GO analysis. In the genotype comparison, the pathway analysis of the Subq AT depot of bGH mice compared to that of WT mice revealed significantly altered pathways in ‘valine, leucine, and isoleucine degradation’ and several pathways related to fatty acid metabolism as well as other metabolic processes and immune cell response (Fig. 7A). Notably, the pathway analysis of the Epi AT depot did not lead to significant results (Fig. 7B). Some of the pathways that were most changed, such as ‘cardiac muscle contraction’ and ‘cardiomyopathy’, were related to significantly altered terms found in the GO cellular component category for the Epi AT depot (Fig. 5C).

Pathway analyses for the AT depot comparisons showed that the ten most significantly altered pathways in both WT and bGH mice were related to immune cell biology (Figs. 7C and 7D). In terms of the bGH mice, immune-related pathways were again significantly altered, but additional pathways include degradation of branched chain amino acids, ‘ECM receptor interaction,’ and carbon and glycerolipid metabolism (Fig 7C).

3.5 The miRNAs mmu-miR-325-3p and mmu-miR-124-3p may be involved in the regulation of lipid and immune metabolism in the AT of bGH mice

The miRNA prediction was based on enrichment of differentially downregulated target genes of the miRNAs. In general, miRNAs have an inhibitory effect on their targets, but based on their sequence, the predicted miRNA could potentially bind to both downregulated and upregulated genes present in the dataset. In terms of the genotype comparison, 467 and 274 miRNAs were predicted in the Subq and Epi AT depots, respectively, in bGH mice compared to controls (suppl. tables 9 and 10). The five miRNAs with the highest number of downregulated genes are shown in figure 8A–B. Interestingly, in the genotype comparison, the two miRNAs with the most downregulated target genes (mmu-miR-325-3p and mmu-miR-124-3p) were the same in both AT depots. To further understand the possible biological significance of these miRNAs, GO analysis for the biological processes was performed on the DEG targeted by each of the miRNAs. A higher number of biological processes were significantly altered in the Subq AT depot than in the Epi AT depot when compared to controls (Fig. 8A). Interestingly, in the WT Subq vs. bGH Subq AT comparison, the miRNAs mmu-miR-325-3p and mmu-miR-182-5p were associated with the ‘T cell proliferation’ and ‘cholesterol biosynthetic’ biological process, respectively. Also, the DEGs targeted by the miRNAs mmu-miR-124-3p and mmu-miR-182-5p were associated with several biological processes related with lipid metabolism (Fig. 8A).

In terms of the AT depot contrast, the same three predicted miRNAs targeted the most downregulated genes (mmu-miR-124-3p, mmu-miR-325-3p and mmu-miR-330-3p) in both WT and bGH mice (Figs. 8C and 8D); note that the first two miRNAs are the same miRNAs with the most targeted DEGs found in the genotype comparison (Figs. 8A and 8B). Also,

when comparing the Epi and Subq AT depots of WT mice, two closely related mature miRNAs, mmu-miR-181b-5p and mmu-miR-181c-5p, were predicted and as expected, targeted the same genes in the DEG list (suppl. table 11). Similar GO-biological process terms associated with miRNAs were found in both genotype and AT depot comparisons. Specifically, the WT Subq vs. bGH Subq comparison (Fig. 8A) and the bGH Epi vs. bGH Subq comparison (Fig 8C and suppl. table 12) showed that the miRNAs mmu-miR-325-3p and mmu-miR-182-5p targeted genes associated with ‘T cell proliferation’ and ‘cholesterol biosynthetic process’, respectively.

4. Discussion

GH is known to radically alter AT. That is, GH reduces AT mass (35), alters adipokine expression (2, 20, 31), increases AT fibrosis (24), increases preadipocyte senescence (22), and modulates AT immune cell infiltration (25, 36), among countless other activities (28). Furthermore, GH's actions on AT are depot dependent (20), so analyzing more than one AT depot is critical to fully understand the impact of GH on AT. Therefore, this study had two main purposes: 1) to investigate the effect of GH excess on AT in a depot specific manner (genotype comparison) and 2) to examine differences between Subq and Epi AT depots in the same mouse line (AT depot comparison). To accomplish these goals, we used RNAseq as an exploratory tool to elucidate the effect of increased GH action in the Subq and Epi AT depots of bGH mice as compared to WT mice. The significantly upregulated expression of IGFBP3, a known GH-regulated gene, in both Subq as well as the Epi depots in bGH mice when compared to corresponding WT depots, corroborates the effect of GH excess on AT (suppl. table 2 and 4). These findings are similar to results reported for Subq AT of patients with acromegaly compared to controls (individuals with non-functioning pituitary adenoma) which also showed an upregulation of genes that are induced by GH action, such as IGFBP3 (37). As might be expected based on many studies that show greater alterations in the Subq AT of bGH mice compared to other depots (24, 31, 38), the bGH mice have more significant DEGs in the Subq AT depot than in the Epi AT depot.

Both genotype and AT depot comparison showed an increased number of DEG in bGH mice. The genotype comparison shows more uniquely expressed genes than shared genes in bGH mice relative to controls, indicating that GH modulates the expression of genes that are not typically or significantly expressed in the AT of WT mice. Moreover, the Epi vs. Subq AT depot comparison reveals that bGH mice have almost a two-fold increase in the number of DEG than WT mice when these two depots are compared. This finding further corroborates that GH excess increases the total number of DEG, in part by impacting the expression of genes that are not normally expressed to a detectable level in the AT of WT mice, and thus, exacerbates the difference between AT depots.

GH is well-known for regulating metabolic homeostasis, including proteins, carbohydrates, and lipids (39). Therefore, it is not surprising that GO analysis reveals that the downregulated genes of the Subq and Epi AT depot of bGH mice are associated with lipid and amino acid metabolism. Likewise, pathway analysis shows that all the significantly altered pathways in the Subq AT depot of bGH mice vs. controls are also related with fatty acid and amino acid metabolism. These results are congruent with the well-established

increase in lipolysis (18, 19) and decrease in lipogenesis caused by GH on AT (17, 19). In terms of the amino acid metabolism, it is interesting that one of the five most significantly altered metabolic processes in the Subq of bGH mice is ‘branched-chain amino acid (BCAA) metabolic process,’ while the BCAA degradation (valine, leucine, isoleucine) pathway is the most affected in the pathway analysis between genotypes. The relationship between BCAAs and GH has been reported previously, with patients with active acromegaly having significantly lower levels of BCAAs, like valine and isoleucine, compared to control groups (40). Interestingly, both GH and BCAAs modulate the mTOR pathway and have been linked to obesity, T2DM, as well as neuropathologies like Alzheimer’s disease (41). At least in muscle, GH appears to modulate the mTOR pathway partially through the stimulated expression of L-type amino acid transporters that delivered BCAAs (42). Although this relationship has garnered little attention in AT, the potential importance between GH function and BCAA metabolism warrants further exploration.

Our GO analysis for the upregulated genes of the Subq AT depot of bGH mice show that the most significantly altered biological processes, molecular functions, and cellular components are related to immune cell biology, especially with leukocyte and T cell activation (Figs. 5B, 6B and 6D). For example, the GO and pathway analyses show that immune cell activation and T cell response may be upregulated in the Subq AT depot of both WT and bGH mice (Figs. 6B and 6D). These results are particularly interesting, because the Subq depot of bGH mice has been shown to have more pronounced alterations in immune cell infiltration (38) and fibrosis (24), promoting a ‘pro-inflammatory’ state in that depot (24). Other studies have implicated the GH/IGF-1 axis in the modulation of the fibrotic and immune response in several tissues. Among other examples, a recent study shows that pharmacologically blocking IGF-1 reduces fibrosis and fat accumulation around the eye (43). Thus, it seems that decreasing GH/IGF-1 activity may reduce the immune response and fibrosis in several tissues, possibly impacting the development and progression of many diseases that involve remodeling of extracellular matrix and immune characteristics.

In agreement with the GO results, numerous genes related to B cell activation (e.g, CD19, CD22, CD79a, and CD79b) were upregulated in both WT and bGH Subq compared to Epi (Fig 4C), indicative of a higher leukocyte infiltration in the Subq depot irrespective of mouse genotype. These findings are also congruent with previous reports in which GH has been shown to increase the percentage of T helper cells in the Subq AT depot of bGH mice (25, 38). It is known that cells from the adaptive immune system, such as T cells and B cells, can be residents of healthy peripheral tissues, including AT (44, 45). Further, AT proximity to lymphoid organs may contribute to the infiltration of T and B lymphocytes into the AT (45), and Subq AT has significant lymph nodes. It is important to remember that the current study performed RNAseq on RNA isolated from whole AT; thus, the changes reported likely reflect shifts in specific cell populations and their cellular activity. While the GO and pathway analyses both imply a change in immune cell population, additional studies on cellular sub-fractions would be needed to verify these results. Other studies have assessed the immune cell population in different AT depots (25, 46). In 2008, Kintscher and colleagues reported that T cells accumulate in the perigonadal AT of mice on HFD even before infiltrating macrophages, and that the accumulation of these T lymphocytes occurs simultaneously with the onset of insulin resistance (46). Therefore, lymphocytes may initiate

the pro-inflammatory and unhealthy phenotype in AT. Consistent with this hypothesis, other studies on six-month-old bGH mice have shown increased extracellular matrix remodeling in the AT (24), as well as alterations in blood pressure (47), and in fat mass when compared to WT controls (31). Interestingly, in 2015, Benencia and colleagues found more cytotoxic T cells in the Epi AT depot than Subq AT depot in bGH mice, and more cytotoxic T cells in Epi AT depot than that of WT controls (25). Thus, it is possible that the pro-inflammatory phenotype of bGH mice begins around six months of age with T lymphocyte infiltration into the tissue. Interestingly, RNAseq data performed in Subq AT of patients with acromegaly show downregulation of genes involved in inflammation (37), which may relate to the species-dependent differences in depot response to GH action noted previously.

The results for miRNA prediction and their targets corroborate previous studies. Lobie and colleagues show that miRNA cluster 96–182–183 is regulated by GH in MCF7 breast cancer cells (48). Accordingly, we observe a highly upregulated expression of miR-182, which, along with miR-325, targets the T-cell proliferation pathway (Fig 8). The differentially expressed miR-96 and miR-182 in the AT depots identified from our analyses also jointly target the sphingosine-1-phosphate receptor (S1PR) signaling, which is a critical mediator of immune modulation by blocking CXCL8 production in activated T-cells (49). S1PR subtypes are expressed in B-cells, T-cells, natural killer (NK) cells, monocytes, macrophages, neutrophils, eosinophils, dendritic cells (DC), and mast cells (50). S1PR activity modulates numerous aspects of the immune system, such as lymphocyte migration from secondary lymphoid organs into circulation, suppressing Treg development, and orchestrating survival, proliferation, and activities of neutrophils, eosinophils, mast cells, DCs, and macrophages (50). The target of differentially expressed miR-181 is found to be the ER-Golgi ceramide transport pathway, which is also a critical step in S1P biosynthesis from sphingomyelin (50). Appropriately, an AT specific dysfunction in S1P signaling also leads to lipodystrophy and insulin resistance (51).

In summary, RNAseq was used to explore the DEG, biological processes, and pathways that may be altered in bGH and WT mice as a function of GH status (WT vs bGH comparison) or AT localization (AT depot contrast). We confirmed that GH excess alters the Subq AT more than the Epi AT depot. Furthermore, we showed that gene expression changes between AT depots are more prominent in bGH mice than in WT mice. Specifically, GH increases the total number of DEG, mainly by increasing the proportion of uniquely expressed genes in the AT of bGH mice. We also found that the DEG of both genotype and depot comparisons are mainly related to lipid metabolism and the immune system (lymphocyte and T cell response). Collectively, these results suggest that excess GH modulates the expression of genes that are not commonly expressed in the AT of WT mice. Moreover, lipid metabolism and immune cell infiltration may help to explain the metabolic effects of AT as a function of both GH action and AT localization in the body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GH	growth hormone
bGH	bovine growth hormone
AT	adipose tissue, adipose tissue macrophages
Subq	subcutaneous
GO	gene ontology
Epi	epididymal
WT	wild type
PCA	Principal component analysis
DEG	differentially expressed genes

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Genotype comparison

AT depot comparison

Figure 2. Heat map of the 20 most significantly altered genes.

A. Downregulated genes for the Subq genotype comparison. **B.** Upregulated genes for the Subq genotype comparison. **C.** Downregulated genes for the Epi genotype comparison. **D.** Upregulated genes for the Epi genotype comparison. **E.** Downregulated genes for the AT depot comparison in bGH mice. **F.** Upregulated genes for the AT depot comparison in bGH mice. **G.** Downregulated genes for the AT depot comparison in WT mice. **H.** Upregulated genes for the AT depot comparison in WT mice. Green denotes downregulated genes and red upregulated genes. *Gene expression expressed in FPKM, significance given with and FDR corrected p value < 0.05.

Figure 3. Shared and divergent genes in the genotype comparison (WT vs. bGH mice).

A. Shared downregulated genes of the Subq WT vs. Subq bGH mice and Epi WT vs. Epi bGH mice comparisons. **B.** Venn diagram of the downregulated genes of the Subq WT vs. Subq bGH mice and Epi WT vs. Epi bGH mice comparisons. **C.** Shared upregulated genes of the Subq WT vs. Subq bGH mice and Epi WT vs. Epi bGH mice comparisons. **D.** Venn diagram of the upregulated genes of the Subq WT vs. Subq bGH mice and Epi WT vs. Epi bGH mice comparisons. **E.** Divergent down- and upregulated genes of the Subq WT vs. Subq bGH mice and Epi WT vs. Epi bGH mice comparisons. Green denotes downregulated genes and red upregulated genes. Numbers in heat maps express log change. *Indicates genes expressed specifically in only one genotype.

Figure 4. Shared and divergent genes in the AT depot comparison (Subq vs. Epi).

A. Shared downregulated genes of the Subq WT vs. Epi WT mice and Subq bGH vs. Epi bGH mice comparisons. **B.** Venn diagram of the downregulated genes of the Subq WT vs. Epi WT mice and Subq bGH vs. Epi bGH mice comparisons. **C.** Shared upregulated genes of the Subq WT vs. Epi WT mice and Subq bGH vs. Epi bGH mice comparisons. **D.** Venn diagram of the upregulated genes of the Subq WT vs. Epi WT mice and Subq bGH vs. Epi bGH mice comparisons. **E.** Divergent down- and upregulated genes of the Subq WT vs. Epi WT mice and Subq bGH v. Epi bGH mice comparisons. Green denotes downregulated genes and red upregulated genes. Numbers in heat maps express log change. **indicates genes expressed specifically in only one AT depot.

Figure 5. Gene ontology (GO) of the DEGs from the genotype comparison.

A. GO of the downregulated genes for the Subq genotype comparison. **B.** GO of the upregulated genes for the Subq genotype comparison. **C.** GO of the downregulated genes for the Epi genotype comparison. **D.** GO of the upregulated genes for the Epi genotype comparison. Bar graphs show the five most significantly enriched GO terms found in the biological process (yellow), molecular function (red) and cellular component (blue) categories. GO was performed using the GO consortium and PANTHER classification system. $p < 0.05$.

Figure 6. Gene ontology (GO) of the DEGs from the AT depot comparison.

A. GO for the downregulated genes for the AT depot comparison in WT mice. **B.** GO for the upregulated genes for the AT depot comparison in WT mice. **C.** GO for the downregulated genes for the AT depot comparison in bGH mice. **D.** GO for the upregulated genes for the AT depot comparison in bGH mice. Bar graphs show the five most significantly enriched GO terms found in the biological process (yellow), molecular function (red) and cellular component (blue) categories. GO was performed using the GO consortium and PANTHER classification system. $p < 0.05$.

Figure 7. Pathway analysis of the DEG.

A. Pathway analysis for the Subq genotype comparison. **B.** Pathway analysis for the Epi genotype comparison. **C.** Pathway analysis for the bGH depot comparison. **D.** Pathway analysis for the WT depot comparison. Bar graphs show the ten most altered biological pathways according to the p value. Pathways were obtained using iPathwayGuide, which scores pathways using two types of data: 1) the over-representation of DEG in a given pathway and 2) the DEG across the pathway topology. The underlying pathway topologies, comprised of genes and their directional interactions, are obtained from the KEGG database. *indicates not significant pathways. $p < 0.05$.

Figure 8. Predicted micro RNAs (miRNAs).

A-D. Bar graphs show the five predicted miRNAs with the highest number of downregulated targets in each comparison. The prediction of active miRNAs was performed using iPathwayGuide and is based on enrichment of DE downregulated target genes of the miRNAs. GO of the biological processes was performed using the GO consortium and Panther classification system. GO analysis results are sorted by the hierarchical relations between over-represented or enriched functional classes. $p < 0.05$.

Table 1.

Comparisons used to analyze the DEGs.

Analysis	Comparisons	#DEGs
Genotype	bGH Subq vs. WT Subq	822
	bGH Epi vs. WT Epi	175
AT depot	WT Subq vs. WT Epi	527
	bGH Subq vs. bGH Epi	1542