Visceral adipose tissue-directed FGF21 gene therapy improves metabolic and immune health in BTBR mice

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Fibroblast growth factor 21 (FGF21) is a peptide hormone that acts on various tissues to maintain energy homeostasis. While FGF21 production predominately occurs in the liver, adipocytes are the main target of FGF21 action. In white adipose tissue (WAT), FGF21 stimulates glucose uptake in an insulin-independent manner, regulates lipolysis, regulates mitochondrial activity, and regulates adaptive thermogenesis. Furthermore, the antidiabetic actions of FGF21—resulting in improvements in obesity-induced hyperglycemia, hypertriglyceridemia, and peripheral insulin resistance—are thought to occur primarily within WAT.

The therapeutic potential of FGF21 is well recognized by the metabolism, pharmaceutical, and gene therapy communities. Increasingly, FGF21 is viewed as a potential therapeutic agent for type 2 diabetes (T2D), fatty liver disease, and other metabolic complications. Exogenous administration of recombinant FGF21 protein to ob/ob, db/db, and high-fat diet (HFD) mice reduces adiposity, lowers blood glucose and triglycerides, and improves insulin sensitivity. Despite early successes, use of native FGF21 peptide was found to be unfavorable due to its short half-life and biophysical deficiencies. While the development of FGF21 analogs and mimetics is ongoing and provides promise, some limitations exist. The use of FGF21 protein analogs or mimetics may require repeated administrations for maintained clinical benefit, which raises concerns about immunological reactions associated with exogenous protein administration, patient comfort, and treatment non-compliance.

Recent work in the field has investigated the use of adeno-associated viral (AAV) vectors for FGF21 gene therapy to treat obesity and insulin resistance. AAV vectors serve as one solution for the troubles of long-term therapeutic protein administration, as they require a single administration for long-term transgene production. AAV vectors are predominately non-integrative, and their genomes persist as episomes in non-dividing cells. Variations in AAV capsids yield tissue tropism, making AAVs adaptable for various therapeutic-, tissue- and cell-specific applications.

WAT is a dynamic endocrine and secretory organ, serving as much more than a mere vessel for energy storage. Visceral adipose tissue (VAT) is a subtype of WAT that surrounds inner organs in the abdominal cavity and is thought to contribute to local/systemic inflammation and metabolic function. Mature adipocytes in VAT depots are terminally differentiated, making them an attractive target for primarily non-integrative gene expression vectors such as AAVs. Recently, we characterized a novel engineered hybrid serotype, Rec2, which achieves superior transduction of adipose tissue when compared to naturally occurring AAV serotypes. We and others have applied Rec2 serotype vectors to manipulate adipose depots of interest in various mouse models. Furthermore, we recently designed a dual-cassette vector to transduce VAT in a highly selective manner, while severely restricting off-target transduction of liver during non-invasive intraperitoneal (i.p.) administration.

Here, we combine these unique delivery systems to investigate the...
potential for WAT-targeted FGF21 gene therapy in obese, insulin-resistant BTBR T+Hpr3tf/J (BTBR) mice.33

RESULTS
To investigate the potential of WAT-directed FGF21 gene therapy to improve metabolic dysregulation, we utilized adipose-targeting recombinant AAV (rAAV)-Rec2 vector containing two expression cassettes, one driving the transgene expression by a constitutive promoter, while the second cassette uses a liver-specific promoter to express a microRNA targeting the woodchuck post-transcriptional regulatory element (WPRE) sequence existing in the first cassette (Figure 1A). i.p. injection of the Rec2 dual-cassette vector achieves selective transduction of visceral adipose depots while severely restricting off-target transgene expression in the liver.25,31,32 This VAT-directed FGF21 gene therapy was applied to insulin-resistant BTBR mice under normal chow diet (NCD) and diet-induced obesity (DIO) conditions.

Intraperitoneal administration of Rec2-FGF21 sustains FGF21 overexpression in VAT and alters hypothalamic gene expression under NCD conditions.
NCD-fed mice were injected i.p. with 2.0 × 10^10 vg of either Rec2-FGF21 or a control vector containing no transgene, Rec2-Empty (Figure 1A). Mice were subjected to various metabolic tests over a 21-week period (Figure 1B). No differences in body weight (Figure 1C) or food intake (Figure 1D) were observed over 21 weeks. An in vivo echoMRI at 4, 7, and 21 weeks post-injection revealed no significant differences in body fat (Figures 1E, S1A, and S1B) or lean mass percentage (Figures 1F, S1C, and S1D). At 5 weeks post-AAV injection, mice were subjected to a glucose tolerance test (GTT) to assess systemic glycemnic processing; no significant differences were observed (Figures 1G and 1H). At 11 weeks post-AAV injection, an open-field (OF) test was performed; no differences in anxiety-like behaviors and locomotion were observed (Figures S1E–S1G). From 12 to 14 weeks post-AAV injection, indirect calorimetry was performed. No significant differences between Rec2-FGF21 and Rec2-Empty groups were observed across various metrics, including VO_{2}, VCO_{2}, respiratory exchange ratio (RER), and ambulation (Figures S2A–S2H). At 20 weeks post-AAV injection, an insulin tolerance test (ITT) revealed no significant differences in non-fasting glucose levels or insulin sensitivity between the two groups (Figures 1I and 1J). At 21 weeks post-injection, tissues and serum were collected. Rec2-FGF21 and Rec2-Empty mice displayed no significant differences in relative tissue mass of brown adipose tissue (BAT), inguinal WAT (iWAT), gonadial WAT (gWAT), retroperitoneal WAT (rWAT), liver, or pancreas (Figure 1K).

Rec2-FGF21-treated mice exhibited an approximately 10-fold elevation of serum FGF21 (Figure 1L). No change in serum leptin (Figure 1M) or chemokine (C-C motif) ligand 2 (CCL2, also known as MCP-1) was observed (Figure 1N). Consistent with serum data, a robust 100-fold upregulation of Fgf21 expression was observed in the gWAT (Figure 1O). Together, these data indicate that Rec2-FGF21 sufficiently up-regulated local and systemic FGF21 levels over the duration of the 21-week study, although no functional changes in systemic metabolism were observed under NCD conditions.

Given that FGF21 acts centrally,34–36 we additionally profiled hypothalamic tissue to assess gene-therapy-induced alterations in neuroendocrine and inflammation markers (Figure S3). FGF21 acts on a receptor complex consisting of the ubiquitously expressed FGF receptor 1 (encoded by Fgfr1) and a co-receptor β-klotho (encoded by Klb) that is restricted to specific metabolic tissues including adipose tissue, liver, and particular areas of brain.37,38 No changes in Fgfr1 and Klb were observed. The Rec2-FGF21-treated group exhibited a trend of up-regulation of Crh (encoding corticotropin-releasing hormone), consistent with peripheral upregulation of FGF21.34 A trend of downregulation of Insr (encoding insulin receptor) was observed in the Rec2-FGF21-treated group. No changes in other neuropeptides or receptors involved in energy balance including Obbr (encoding leptin receptor), Npy (encoding neuropeptide Y), Pomc (encoding proopiomelanocortin), or Trkb-FL (encoding full-length tropomyosin receptor kinase B) were observed. Interestingly, a bevy of inflammatory cytokines and immune modulatory genes—including Ccl2, Il1b (encoding interleukin-1β), Ilkbb (encoding inhibitor of nuclear factor kappa-B kinase subunit beta), Tnfα (encoding tumor necrosis factor alpha), Il33 (encoding interleukin-33), and H2ab1 (encoding histocompatibility 2, class II antigen A, beta)—were collectively downregulated in the hypothalamus of the Rec2-FGF21 group.

VAT-directed overexpression of FGF21 improves systemic metabolism in DIO mice
Following 4 weeks of HFD feeding, a separate DIO cohort of mice was injected i.p. with 2.0 × 10^10 vg of either Rec2-FGF21 or Rec2-Empty. Mice were subjected to various metabolic tests over a 16-week period (Figure 2A). No differences in absolute body weight were observed over the course of the experiment (Figure 2B). While Rec2-Empty mice continued to gain weight, we observed a moderation in DIO-induced weight gain in Rec2-FGF21-injected mice (Figure 2C). In tandem with these observations, Rec2-FGF21 mice exhibited increased relative food consumption calibrated to body weight, suggesting an increase in energy expenditure (Figure 2D). An in vivo echoMRI revealed Rec2-FGF21 mice to have a reduced body fat percentage (Figures 2E and S4A) and increased lean mass percentage (Figures 2F and S4B) when compared to Rec2-Empty controls. At 8 weeks post-AAV injection, mice were subjected to an ITT. Rec2-FGF21 mice showed significantly lower non-fasting blood glucose levels (at t = 0) and improved overall response to the ITT (Figures 2G and 2H), indicative of alleviation of insulin resistance in the obese BTBR mouse model. At 10 weeks post-AAV-injection, an OGTT test was performed; no differences in anxiety-like behaviors and locomotion were observed (Figures S4C–S4E). At 10 weeks post-AAV-injection, a GTT was performed. Rec2-FGF21 mice cleared an i.p. glucose bolus in a more efficient manner, indicating an improvement in glycemnic processing following FGF21 gene therapy (Figures 2I and 2J).

From 11 to 14 weeks post-AAV-injection, mice were subjected to indirect calorimetry. Rec2-FGF21 mice exhibited an increased oxygen
Figure 1. VAT-directed Rec2-FGF21 gene therapy under NCD conditions
(A) Vector schematic for Rec2-Empty and Rec2-FGF21. (B) Experimental timeline. (C) Body weights following rAAV administration. (D) Relative daily food intake. (E) Relative fat mass at 21 weeks post-rAAV-injection. (F) Relative lean mass at 21 weeks post-rAAV-injection. (G) Glucose tolerance test at 5 weeks post-rAAV-injection. (H) Glucose tolerance test area under the curve. (I) Insulin tolerance test at 20 weeks post-rAAV-injection. (J) Insulin tolerance test area under the curve. (K) Relative tissue weight at sacrifice. (L) Serum FGF21 at sacrifice. (M) Serum leptin at sacrifice. (N) Serum CCL2 at sacrifice. (O) Gene expression profile of gWAT (n = 4, Rec2-Empty; n = 6, Rec2-FGF21). Data are means ± SEM. Sample size, Rec2-Empty, n = 4; Rec2-FGF21, n = 8 unless otherwise noted. +p < 0.06, *p < 0.05, **p < 0.01, ***p < 0.001.
consumption (Figures 3A and 3B) and carbon dioxide expiration (Figures 3C and 3D), indicative of elevated energy expenditure consistent with previous observations following FGF21 gene therapy.\textsuperscript{16} No differences were observed in RER (Figures 3E and 3F) or ambulation (Figures 3G and 3H). Together, these data indicate FGF21 gene therapy to the VAT resulted in improved systemic metabolism.

At 16 weeks post-AAV-injection, mice were sacrificed and tissues were collected. Rec2-FGF21 mice displayed reduced iWAT weight
No differences were observed in the tissue weight or relative tissue weight of the virally treated gWAT (Figures 4A and 4B). Rec2-FGF21 mice additionally displayed a significant increase in relative tissue weight of gastrocnemius (Figure 4B). A large reduction in liver absolute weight and relative tissue weight was observed in the Rec2-FGF21 group (Figures 4C and 4D). Hepatic steatosis was alleviated in Rec2-FGF21 mice as measured by liver H&E staining (Figure 4E) and triglyceride quantification (Figure 4F). Of note, Fgf21 overexpression was not observed in the liver, consistent with the liver-restricting nature of the dual-cassette AAV vector design (Figure 4G).

**VAT-directed FGF21 gene therapy alters serum adipokine and inflammation markers in DIO mice**

As expected, FGF21 was increased in the serum of Rec2-FGF21 mice (Figure 5A). Given this observation, we profiled various serum markers of metabolic function and inflammation to assess changes following FGF21 gene therapy.

Adiponectin is an adipokine that has been shown to connect FGF21 action in adipocytes to liver and skeletal muscle, thus improving insulin sensitivity, glucose homeostasis, and systemic metabolism.2,39 Accordingly, there was a trend toward increased total adiponectin (p = 0.08) (Figure 5B) and significantly increased high-molecular-weight (HMW) adiponectin in the serum of Rec2-FGF21 mice (Figure 5C). The ratio of HMW adiponectin to total adiponectin has been described as an advanced marker of systemic metabolism and cardiac health.40 Rec2-FGF21 mice displayed an increase in the HMW:total adiponectin ratio (Figure 5D).

Leptin is predominantly secreted by adipocytes and serves as a central-peripheral messenger to maintain energy homeostasis. Leptin production is positively correlated with adipose tissue mass and additionally has been described as a proinflammatory link between immune and neuroendocrine systems.41 Rec2-FGF21 mice displayed approximately 70% reduction of serum leptin level (Figures 5C and 5E). No changes in serum fatty acids (Figure 5F), triglycerides (Figure 5G), or glucose (Figure 5H) were observed. A trending, but not significant (p = 0.08), decrease in serum insulin was observed (Figure 5I), indicative of a trend toward improved insulin sensitivity. No difference was observed in the HOMA-IR (homeostasis model assessment of insulin resistance) index of the two groups after a 4-h fast (Figure 5J).

We additionally profiled several serum proinflammatory cytokines and chemokines. Serum amyloid A (SAA) serves as a marker of inflammation and is thought to be tied to macrophage-related immunologic pathways.42,43 We observed a strong trend of reduction of
SAA in the Rec2-FGF21 group as compared to controls (Figure 5K). Plasminogen activator inhibitor-1 (PAI-1) is involved in fibrinolysis and its elevation thought to contribute to vascular disease and inflammation in obese states.\(^4\)\(^4\)\(^5\) PAI-1 levels were reduced in the Rec2-FGF21 group (Figure 5L), indicative of improved metabolic function and reduced inflammation. Serum CCL2 levels were reduced in the Rec2-FGF21 group (Figure 5M), indicative of decreased inflammation; these findings are additionally consistent with the observed improvements in insulin sensitivity.\(^4\)\(^6\) No change in interleukin-1 beta (IL-1\(\beta\)) serum levels was observed (Figure 5N).

VAT-directed FGF21 gene therapy alters adipose and hypothalamic gene expression in DIO mice

In the gWAT, Fgf21 was robustly overexpressed, with Rec2-FGF21-treated tissue showing a 40-fold increase over Rec2-Empty controls (Figure 6A). This overexpression was accompanied by a downregulation of FGF21 receptor genes, Fgfr1 and Klb (Figure 6A). No significant differences were observed in two other FGF21 receptors, Fgfr2 and Fgfr3. A significant downregulation of Lep (encoding leptin) was observed in Rec2-FGF21-treated gWAT, consistent with serum observations. No change in Adipoq (encoding adiponectin) was observed. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-\(\alpha\), encoded by Ppargc1a), a transcriptional coactivator involved in mitochondrial biogenesis,\(^4\)\(^7\) was significantly upregulated in Rec2-FGF21-treated gWAT, while no change in Ucp1 (encoding uncoupling protein-1) was observed (Figure 6A). In addition, several markers of inflammation were assessed in the gWAT. Cd2 mRNA remained unchanged (Figure 6A), despite reduced serum levels. Pai1 expression was reduced in the Rec2-FGF21 group, consistent with serum observations (Figure 6A). Two markers of inflammation involved in the NLRP3 inflammasome complex, Casp1 (encoding caspase-1) and PyCARD (encoding apoptosis-associated speck-like protein containing a CARD) were reduced in Rec2-FGF21-treated gWAT. No changes in macrophage markers Il10 (encoding interleukin-10), Arg1 (encoding arginase), Mrc1 (encoding mannose receptor c-type 1), or Clec10a (encoding c-type lectin domain family 10 member A) were observed in the gWAT (Figure 5A).

We assessed gene expression in an additional VAT depot, the rWAT (Figure 6B). A robust 120-fold overexpression of Fgf21 was observed in the Rec2-FGF21-treated rWAT. Despite this robust overexpression, no changes were observed in various adipokine and inflammation markers. In the iWAT—a nontargeted subcutaneous adipose depot—no changes in Fgf21 or its receptors were observed (Figure 6C), consistent with the viral administration technique. In the iWAT, adipokine and mitochondrial markers remained unchanged.

Figure 4. Gross tissues and liver analysis

(A) Tissue weights at sacrifice. (B) Relative tissue weights at sacrifice. (C) Liver weight at sacrifice. (D) Relative liver weight at sacrifice. (E) Representative H&E staining of liver tissue. Scale bar, 50 \(\mu\)m. (F) Hepatic triglycerides. (G) Gene expression profiling of liver tissue. Data are means ± SEM. Sample size, Rec2-Empty, n = 7; Rec2-FGF21, n = 7. +p < 0.06, *p < 0.05, **p < 0.01, ***p < 0.001.
Two markers of inflammation, Ccl2 and Ptnl, were downregulated in the Rec2-FGF21 group (Figure 6C). In the BAT, gene expression of Fgf21 and its receptors remained unchanged (Figure 6D). Rec2-FGF21 mice displayed a reduction of Lep gene expression in the BAT, with no change in Adipoq. In the BAT, various markers of mitochondrial function, thermogenesis, and fatty acid synthesis/oxidation—including Ppargc1a, Ucp1, Dio2 (encoding iodothyronine deiodinase 2), and Ppara (encoding peroxisome proliferator activated receptor alpha)—remained unchanged (Figure 6D). No transgene expression was found in pancreas or skeletal muscle. No changes in Fgf21, Fgfr1, or Klb were observed in the pancreas (data not shown) or the gastrocnemius (Figure S5B).

It is well documented that FGF21 acts centrally—through CRH—to induce energy expenditure, thermogenesis, and sympathetic nerve activity. However, in this study, we observed upregulation of Tnfa in the Rec2-FGF21 group.

**VAT-directed FGF21 gene therapy reduces ATM inflammation in DIO mice**

Under obese conditions, adipose tissue macrophages (ATMs) accumulate in VAT and exhibit a proinflammatory M1 polarization (CD11c+, CD206−), contributing to insulin resistance. In contrast, lean animals present primarily with an M2-polarized state (CD11c−, CD206+), which is thought to protect adipocytes from inflammation. As such, we isolated the stromal vascular fraction (SVF) from gWAT and performed fluorescence-activated cell sorting (FACS). Administration of Rec2-FGF21 resulted in distinct changes in ATM polarization within the VAT (Figure 7A). While no percentage changes in the total population of ATMs (Figure 7B) and M1-polarized populations (Figure 7C)
and insulin resistance in HFD and highlighted the potential for FGF21 gene therapy to counter obesity gene expression. This technique builds upon an extensive report that VAT-specifically targeted administration of FGF21 reduced ATM in liver and heart.49 We used an engineered hybrid serotype Rec2 vector—which transduces adipose tissue more efficiently than the naturally occurring AAV8 and— in combination with a dual-cassette design to restrict off-target liver transduction.45 This study adds to the literature that characterizes the efficacy of this vector system to selectively transduce adipose tissue.40-42 Second, Jimenez and colleagues43 performed a laparotomy to directly administer their AAV8 vectors to VAT, whereas the technique presented here allows for non-invasive i.p. injections. In theory, our technique is clinic friendly and stresses the importance of developing minimally invasive administration techniques for widespread use of AAVs as therapeutic agents.

Functionally, our vectors performed similarly. VAT-specific overexpression of FGF21 in ob/ob and BTBR mouse models resulted in increased serum FGF21, increased serum adiponectin, improved glycemia and insulinemia, improved insulin sensitivity, and reduced hepatic steatosis. The previous work found that FGF21 gene therapy to VAT reduced immunostaining of a macrophage marker, Mac2, and expression of F4/80.46,47 Our work expands upon these observations, as we performed FACS to more comprehensively observe the immune populations residing in the AAV-treated gWAT, thus profiling ATM polarization and T cell subsets. Importantly, we discovered Rec2-FGF21 gene therapy altered ATM polarization toward a less-inflammatory phenotype, characterized by an increase in anti-inflammatory M2 polarization (CD11c+CD206+) and a decrease in proinflammatory double-positive (CD11c+CD206+) ATM expression. These ATM phenotypes have been shown to have causal links to lean states and improved insulin sensitivity.48,49 Consistent with the favorable ATM polarization, Rec2-FGF21 administration downregulated the expression of proinflammatory cytokines, chemokines, and inflammasome components in treated gWAT. This gene signature was associated with significant reduction of cytokine and chemokine levels in circulation, suggesting alleviating VAT inflammation is sufficient to lessen systemic chronic inflammation—which, importantly, is implicated in various diseases beyond obesity and T2D. These observations warrant further investigation of VAT-targeted FGF21 gene therapy to treat cardiovascular diseases, non-alcoholic fatty liver disease, and certain types of cancer.

Adiponectin has been proposed as a messenger that links FGF21 actions in local adipocytes to liver and skeletal muscle.50,51 FGF21 was observed, a significant percentage increase in M2 polarization was observed in VAT of Rec2-FGF21 mice (Figure 7D). This change was accompanied by a significant percentage decrease in double-positive (CD11c+, CD206+) ATMs (Figure 7E). ATMs with this signature have been identified as sources of proinflammatory cytokines and are thought to be drivers of insulin resistance.48 These data indicate that FGF21 gene therapy to the VAT reduced ATM inflammation and is associated with the observed improvements in insulin sensitivity and systemic metabolism. We additionally profiled other immune cell populations in the gWAT. No changes were observed in populations of natural killer T cells (Figure 7F), T cells (Figure 7G), or subpopulations of CD4+ T cells (Figure 7H) and CD8+ T cells (Figure 7I).

**DISCUSSION**

The present work provides evidence of a novel adipose-targeting, liver-restricting rAAV vector for long-term, specific transgene expression within VAT. Here, VAT depots were targeted using the adipotrophic Rec2 serotype in tandem with a dual-cassette rAAV utilizing a liver-restricting element.25 Combined, these techniques allow for a minimally invasive delivery system that is equivalent to direct fat injections. By utilizing the VAT as an FGF21 "factory" or "pump" to induce FGF21 in circulation, insulin resistance and obesity were reversed in BTBR mice. In additional, local and systemic obesity-associated inflammation was reduced.

Here, we report i.p. Rec2-FGF21 administration promotes a robust VAT-specific overexpression of FGF21 with no change in liver transgene expression. This technique builds upon an extensive report that highlighted the potential for FGF21 gene therapy to counter obesity and insulin resistance in HFD and ob/ob murine models.16 Our technique differs in two important manners. First, the previous report used AAV8 serotype vector and target sequences for miR-122a and miR-1 to limit transgene expression in the liver and heart.16 We used a dual-cassette design to restrict off-target liver transduction.25,31,32 Secondly, Jimenez and colleagues33 performed a laparotomy to directly administer their AAV8 vectors to VAT, whereas the technique presented here allows for non-invasive i.p. injections. In theory, our technique is clinic friendly and stresses the importance of developing minimally invasive administration techniques for widespread use of AAVs as therapeutic agents.

Functionally, our vectors performed similarly. VAT-specific overexpression of FGF21 in ob/ob and BTBR mouse models resulted in increased serum FGF21, increased serum adiponectin, improved glycemia and insulinemia, improved insulin sensitivity, and reduced hepatic steatosis. The previous work found that FGF21 gene therapy to VAT reduced immunostaining of a macrophage marker, Mac2, and expression of F4/80.16 Our work expands upon these observations, as we performed FACS to more comprehensively observe the immune populations residing in the AAV-treated gWAT, thus profiling ATM polarization and T cell subsets. Importantly, we discovered Rec2-FGF21 gene therapy altered ATM polarization toward a less-inflammatory phenotype, characterized by an increase in anti-inflammatory M2 polarization (CD11c+CD206+) and a decrease in proinflammatory double-positive (CD11c+CD206+) ATM expression. These ATM phenotypes have been shown to have causal links to lean states and improved insulin sensitivity.48,49 Consistent with the favorable ATM polarization, Rec2-FGF21 administration downregulated the expression of proinflammatory cytokines, chemokines, and inflammasome components in treated gWAT. This gene signature was associated with significant reduction of cytokine and chemokine levels in circulation, suggesting alleviating VAT inflammation is sufficient to lessen systemic chronic inflammation—which, importantly, is implicated in various diseases beyond obesity and T2D. These observations warrant further investigation of VAT-targeted FGF21 gene therapy to treat cardiovascular diseases, non-alcoholic fatty liver disease, and certain types of cancer.

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Treatment increases adiponectin secretion from adipocytes, and adiponectin has been shown to promote M2 macrophage polarization. Additionally, adiponectin has been shown to confer the effects of FGF21 on hepatic fatty acid oxidation and lipid clearance. Interestingly, we observed reduced hepatic steatosis following VAT-directed gene therapy; this change was not due to increased hepatic FGF21 expression. We observed increased serum adiponectin following VAT-directed FGF21 gene therapy, providing one potential explanation for the observed reduction in hepatic triglycerides. While not the primary focus of this work, these findings highlight the importance of investigating tissue crosstalk following tissue-directed gene therapy and understanding mechanistic players in such processes.

In the BAT of the Rec2-FGF21-treated group, we observed no change in Ucp1 expression or associated genes. Previous work has shown that FGF21 increases whole-body energy expenditure in ablated BAT and UCP-1 knockout mouse models, suggesting FGF21 plays a role in UCP-1-independent thermogenic processes. In contrast to our data, the previous report showed that liver-directed administration of FGF21 gene therapy induced UCP-1 and browning in BAT. Further work is needed to understand the mechanisms of FGF21 in UCP-1-dependent/-independent thermogenesis and to delineate whether tissue source of FGF21 (e.g., liver or adipose tissue) matters in such biological processes.

WAT depots vary in function and their responses to metabolic stimuli. WAT is broadly classified into two depots—VAT and subcutaneous adipose tissue. The former surrounds internal organs and is associated with insulin resistance, metabolic disease, and is thought to contribute to local/systemic inflammation. The latter is found predominantly around the thighs and is associated with insulin sensitivity. In the present work, we target VAT preferentially with Rec2-FGF21. i.p. administration of Rec2 vector did not result in transgene expression in subcutaneous iWAT. Interestingly, mild reductions in inflammation and relative tissue weight were observed in iWAT in absence of increased Fgf21, Fgfr1, and Klb expression. These results bring questions regarding adipose-adipose crosstalk and FGF21’s role in mediating overall metabolic function. FGF21 has differential actions on various WAT depots; in subcutaneous adipose tissue, FGF21 regulates PGC1-α and browning in adaptive thermogenesis. Furthermore, a recent report suggests that FGF21 induces transcriptomic changes associated with reduced subcutaneous adipose tissue weight. Data on FGF21’s specific roles in VAT are less conclusive and warrant further investigation. The AAV technology presented here provides one such method to further probe these depot-specific roles; two visceral adipose depots, gWAT and rWAT, displayed robust transgene expression with negligible transgene expression in subcutaneous iWAT. Interestingly, mild reductions in inflammation and relative tissue weight were observed in iWAT in absence of increased Fgf21, Fgfr1, and Klb expression. These results bring questions regarding adipose-adipose crosstalk and FGF21’s role in mediating overall metabolic function. FGF21 has differential actions on various WAT depots; in subcutaneous adipose tissue, FGF21 regulates PGC1-α and browning in adaptive thermogenesis. Furthermore, a recent report suggests that FGF21 induces transcriptomic changes associated with reduced subcutaneous adipose tissue weight. Data on FGF21’s specific roles in VAT are less conclusive and warrant further investigation. The AAV technology presented here provides one such method to further probe these depot-specific roles; two visceral adipose depots, gWAT and rWAT, displayed robust transgene expression with negligible transgene expression in subcutaneous iWAT. Notably, growing evidence suggests incongruences between functional aspects of human and murine WAT depots—careful experimental design and depot-specific techniques must be used to aid in translation of murine findings to human health.
BTBR mice are an inbred strain often used as a model of autism spectrum disorder (ASD). HFD is shown to exacerbate social deficiencies and cognitive rigidity in BTBR mice. Moreover, BTBR mice display aberrant immune responses compared to more sociable C57BL/6 mice, characterized by higher anti-brain antibodies, elevated expression of cytokines in the brain—particularly IL-33 and IL-18, and an increased proportion of MHC II-expressing microglial cells. It is proposed that the constitutive neuroinflammation indicates an autoimmun e profile contributing to their aberrant behaviors. Rec2-FGF21 treatment led to downregulation of a cluster of immune-modulatory genes in the hypothalamus (Il13, Il1b, Ccl2, Tnfa, Ikkb, H2Ab1; Figure S3) under NCD conditions, although metabolic outcomes were unremarkable. The impact of adipose-targeted FGF21 treatment on neuroinflammation, behaviors, and the underlying mechanisms warrant future investigation. Another unexpected finding is that the HFD-induced hypothalamic neuroinflammation in C57BL/6 mice was absent in the BTBR mice, although BTBR mice remain more prone to DIO. These observations warrant further work on (1) the aberrant neuroendocrinological and neuroimmunological differences between the insulin-resistant, ASD-like BTBR model and sociable strains, and (2) assessments of the potential benefits of FGF21 treatment beyond metabolic outcomes.

From a therapeutic standpoint, FGF21 AAVs provide several advantages to administration of native FGF21 peptide, analogs, and/or mimetics; such therapeutics require repeated administration, patient adherence, and may be subject to immunological concerns stemming from use of exogenous proteins. In contrast, FGF21 gene therapy via AAV constructs would require but a single administration for long-term transgene persistence. AAV-FGF21 vectors have the additional advantage of producing the wild-type protein, which is easily recognized by canonical FGF21 signaling pathways and has additional advantage of producing the wild-type protein, which is easily recognized by canonical FGF21 signaling pathways and has a reduced likelihood of inducing peptide-related adverse immune responses.

It is important to consider the use indications for FGF21 gene-therapy vectors, analogs, and mimetics. Importantly, we observe limited alterations in systemic metabolism following VAT-directed FGF21 gene therapy in mice on NCD. At this time, the use of AAV FGF21 techniques would not be indicated for use in non-obese individuals like the ones reported in the NCD study (Figure 1). Indeed, the overwhelming majority of preclinical and clinical trials for FGF21 gene therapy, analogs, and mimetics are for obese and/or diabetic individuals. Some have considered aging as an indication for FGF21 therapy, analogs, and mimetics; such therapeutics require repeated administration, patient adherence, and may be subject to immunological concerns stemming from use of exogenous proteins. In contrast, FGF21 gene therapy via AAV constructs would require but a single administration for long-term transgene persistence. AAV-FGF21 vectors have the additional advantage of producing the wild-type protein, which is easily recognized by canonical FGF21 signaling pathways and has a reduced likelihood of inducing peptide-related adverse immune responses.

In summary, AAV-mediated gene therapy is increasingly attractive as a strategy to fight obesity and metabolic diseases. Excessive adiposity is a risk factor for T2D, metabolic syndrome, inflammation, and certain types of cancer. VAT is a prime therapeutic target due to its nature as a secretory organ; adipokines can be harnessed to induce local and systemic improvements in metabolic and immune health. Our study combines an engineered AAV serotype, liver-restricting design, and i.p. administration techniques to provide an example of VAT-targeted gene therapy. Currently, the vast majority of peripheral gene therapies target liver or muscle; the advantages and drawbacks of using these tissues as targets are well characterized. In contrast, the advantages and disadvantages of adipose tissue as a targeting tissue remain largely unknown and warrant further investigation. The recent development of AAV vectors with improved adipose tropism and restriction of off-target transduction paves ways to investigate the long-term transgene expression, local and systemic immune responses, therapeutic efficacy, and safety profile of these vectors in adipose tissue. New AAV administration techniques and bioengineering projects will be essential to increase specificity and efficacy of targeted gene therapies.

MATERIALS AND METHODS

Animals
BTBR (Jackson Laboratory #002282) mice were obtained and bred in-house. Mice were housed in temperature (22°C – 24°C) and humidity (30%–70%) controlled rooms under a 12-h:12-h light:dark cycle. All animal experiments were in accordance with the regulations of The Ohio State University’s Institutional Animal Care and Use Committee.

NCD mice
Adult male BTBR mice (16–20 weeks old) were placed on NCD (11% fat, caloric density 3.4 kcal/g, Teklad). At baseline, mice were randomly assigned to create two groups (n = 4, Rec2-Empty; and n = 8, Rec2-FGF21) that had no significant differences in age, body weight, fat mass percentage, or lean mass percentage. Following randomization, mice were administered rAAV vectors as described below. Mice were maintained on NCD for the remainder of the study, having ad libitum access to food and water. Body weights were monitored on a weekly basis. In vivo measurements occurred according to the timeline in Figure 1B.

DIO mice
Adult male BTBR mice (13–19 weeks old) were placed on HFD (60% kcal from lard; Research Diets #D12492). After 4 weeks of HFD, mice were randomized to create two groups (n = 7, Rec2-Empty; and n = 7, Rec2-FGF21) that had no significant differences in age, body weight, fat mass percentage, or lean mass percentage. Following randomization, mice were administered rAAV vectors as described below. Mice were maintained on HFD for the remainder of the study, having ad libitum access to food and water. Body weights were monitored on a weekly basis. In vivo measurements occurred according to the timeline in Figure 2A.
rAAV design and administration
Adipo-trophic rAAV serotype Rec2 vectors contained two expression cassettes. The first cassette consisted of the cytomegalovirus (CMV) enhancer and chicken β-actin (CBA) promoter, FGF21 transgene, WPRE, and bovine growth hormone poly(A) (Rec2-FGF21; Figure 1A). The second cassette encoded a microRNA targeting the WPRE sequence driven by basic albumin promoter to limit transgene expression in the liver. This liver-restricting dual cassette was previously described and verified. The empty control vector (Rec2-Empty) lacked a transgene insertion in the multiple cloning sites. Rec2 serotype specificity, transduction efficacy in adipose tissue, and packaging were previously detailed elsewhere. Rec2-Empty and Rec2-FGF21 rAAV vectors (2 × 10^10 vg) were administered to mice via i.p. injections (in 150 µL AAV buffer).

EchoMRI
EchoMRI was utilized to measure body composition of fat and lean mass in live mice without anesthesia. Body composition analysis was performed with an echoMRI 3-in-1 analyzer at the Small Animal Imaging Core of the Dorothy M. Davis Heart & Lung Research Institute, The Ohio State University. Fat, lean, free water, and total water mass were measured by the echoMRI machine and then normalized to total body weight as measured 10 min prior to the scan.

ITT
Mice were injected i.p. with an insulin solution (1.5 U insulin per kg body weight) under non-fasting conditions. Blood was obtained from the tail at baseline, 15, 30, 60, 90, and 120 min after insulin injection. Blood glucose concentrations were measured with a portable glucose meter (Bayer Contour Next).

OF test
Mice were individually placed into the center of an open square arena (60 × 60 cm, enclosed by walls of 48 cm). Each mouse was allowed to explore the arena for 10 min, during which time and locomotion—in the center and the periphery of the OF—was recorded and analyzed via TopScan (CleverSys) software. Between each trial, the arena was cleaned with Opticide to remove odor cues.

GTT
Mice were injected i.p. with glucose solution (1.0 g glucose per kg body weight) after a 17-h overnight fast. Blood was obtained from the tail at baseline, 15, 30, 60, 90, and 120 min after glucose injection. Blood glucose concentrations were measured with a portable glucose meter (Bayer Contour Next).

Indirect calorimetry
Mice underwent indirect calorimetry using a comprehensive laboratory animal monitoring system (CLAMS; Columbus Instruments, Columbus, OH, USA). Mice were singly housed and had ample access to HFD and water. In our experience, BTBR mice have difficulty using novel water lixits. As such, mice were additionally supplemented with HydroGel cups (Clear H2O #70-01-5022). Mice were allowed to habituate for 16–18 h and then various physiological and behavioral parameters (VO2, VCO2, RER, heat, and ambulation) were recorded at room temperature for 24 h. Mice were returned to their home cage after indirect calorimetry was performed.

Food intake
For the NCD experiment mice, weekly food intake was measured at the cage level and normalized to body weight and the number of mice per cage. Due to worries of food loss stemming from the physical consistency of HFD, the HFD mice were singly housed for 72 h. Food intake was measured every 24 h to provide three replicate measurements of daily intake per mouse. Measurements were normalized to body weight. HFD mice were returned to their home cages following food intake assessments.

Serum and tissue collection
Mice were euthanized and tissues were collected at 21 weeks post-injection (NCD mice) and 16 weeks post-injection (HFD mice). Trunk blood was collected at 10:00 following a 4-h fast. Blood was allowed to clot on ice for at least 30 min before centrifugation at 10,000 rpm for 10 min at 4°C. The serum component was collected and stored at −20°C until further analysis. Tissues were either fixed as described below or flash frozen and stored at −80°C until further analysis. Fat depots were identified, collected as described elsewhere, and normalized to body weight as measured 10 min prior to euthanasia.

Histology
At sacrifice, portions of liver and adipose tissues were fixed in 10% formalin (w/v) for 48–72 h and then dehydrated with 70% ethanol. Tissues were embedded in paraffin, sectioned, and H&E stained by the Comparative Pathology and Mouse Phenotyping and Histology/Immunohistochemistry (CPMPSR) core of The Ohio State University Comprehensive Cancer Center. Tissue sections were imaged at 20× magnification using an Olympus BX43 microscope with an Olympus SC30 color camera attachment and Olympus cellSens software.

Hepatic triglyceride quantification
Lipids were extracted from liver tissue by chloroform/methanol (2:1 v/v), followed by rinses in 50 mM NaCl and CaCl2 (0.36 M)/methanol (1:1 v/v). Hepatic triglyceride quantification was performed with a Caymen Chemical triglyceride assay kit (#10010303).

Quantitative real-time PCR
Total RNA was isolated from adipose tissue using the RNeasy lipid kit plus RNase-free DNase treatment (QIAGEN #74804). First-strand cDNA was generated using TaqMan reverse transcription reagent (Applied Biosystems #N8080234). Quantitative real-time PCR was performed using power SYBR green PCR master mix (Applied Biosystems #A25742) on a StepOnePlus real-time PCR system (Applied Biosystems). Primers were designed to detect the following genes: Adipoq, Arg1, Casp1, Ccl2, Clec10a, Crh, Dio2, Fgf21, Fgfr1, Fgfr2, Fgfr3, H2Ab1, Ibkbkb, Il10, Il18, Il1b, Il33, Insr, Klb, Lep, Mrc1, Npy,
Obrb, Pati1, Pomc, Ppara, Ppargca1a, Pycard, Tnfα, TrkB-FL, and Ucp1 (Table S1). Data were calibrated to endogenous control Actb or 36B4 (adipose), Ppia (liver), Hp1t (hypothalamus), Gapdh (muscle), and the relative gene expression was quantified using the 2^–ΔΔCT method.81

Serum analysis
R&D Systems DuoSet ELISA kits were used to assay serum FGF21 (#DY3057), leptin (#DY498), SAA (#DY2948), PAI-1 (#DY3828), CCL2 (#DY479), and IL-1β (#DY401). Cayman Chemical assay kits were used to assay serum triglycerides (#10010303), free fatty acids (#700310), and glucose (#10009582). ALPCO ELISA kits were used to assay total/HMW adiponectin (#47-ADPMS-E01) and insulin (#80-INSMSU-E01). HOMA-IR index was calculated as (fasting serum glucose [mmol/L] × fasting serum insulin [pmol/L])/22.5 as described elsewhere.82

Isolation of adipose SVF and FACS
Samples of gWAT were minced into small pieces in Kreb-Ringer HEPES buffer (pH 7.4). Collagenase (1 mg/mL, Sigma #C6885) was added and incubated for 40 min at 37°C with shaking. The mixture was centrifuged to separate the floating adipocytes from the SVF. The SVF pellet was treated with ammonium chloride solution to lyse the red blood cells, then washed and resuspended in FACS buffer. 20 min. The antibodies used for flow cytometry immunophenotyping are listed in Table S2. Cell events were acquired using LSRII (BD Biosciences), and the results were analyzed using FlowJo software (Tree Star).

Statistical analysis
Data are expressed as mean ± SEM. GraphPad Prism 7 software (GraphPad, La Jolla, CA, USA) and SPSS Statistics v25.0.0.0 (IBM, Armonk, NY, USA) were used to analyze data. Student’s t tests were performed for all data except time course data. Mixed-model ANOVAs were used to analyze time course data (weekly body weights, GTTs, ITTs, and indirect calorimetry time measurements). Results of the between-group analyses are reported in the associated figures for weekly body weights and indirect calorimetry time measurements. Results of the pairwise comparisons are reported in the GTT and ITT graphs.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.12.011.

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AUTHOR CONTRIBUTIONS
N.J.Q. and R.B. designed the studies, carried out the research, interpreted the results, and wrote the manuscript. W.H. designed the studies, carried out the research, and interpreted the results. R.X. and B.A. carried out the research and interpreted the results. L.C. conceived the concept, designed the research, interpreted the results, and wrote the manuscript.

DECLARATION OF INTERESTS
L.C. and W.H. are inventors of a provisional patent application related to the liver-restricting AAV vector. All other authors declare no conflicts of interest.

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