Dietary Intake Regulates White Adipose Tissues Angiogenesis via Liver Fibroblast Growth Factor 21 in Male Mice

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Abbreviations: BAT, brown adipose tissue; Dio2, iodothyronine deiodinase 2; Elovl6, ELOVL family member 6, elongation of very long chain fatty acids; eWAT, epididymal white adipose tissue; FGF21, fibroblast growth factor 21; FGF21 LKO, fibroblast growth factor 21 liver-specific knockout; GTT, glucose tolerance test; HFD, high-fat diet; IF, intermittent fasting; IL-1β, interleukin 1β; iWAT, inguinal white adipose tissue; Pgc1α, peroxisome proliferator activated receptor gamma coactivator 1-alpha; TNFα, tumor necrosis factor α; UCP1, uncoupling protein 1; VEGF, vascular endothelial growth factor; WAT, white adipose tissues.

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Abstract

Obesity and related metabolic disorders have become epidemic diseases. Intermittent fasting has been shown to promote adipose tissue angiogenesis and have an anti-obesity feature; however, the mechanisms of how intermittent fasting modulates adipose tissues angiogenesis are poorly understood. We investigated the effect of fasting on vascular endothelial growth factor (VEGF) levels in white adipose tissues (WAT) and the function of fibroblast growth factor 21 (FGF21) in 1-time fasting and long-term intermittent fasting-induced VEGF expression. In the current study, fasting induced a selective and drastic elevation of VEGF levels in WAT, which did not occur in interscapular brown adipose tissue and liver. The fasting-induced Vegfa expression occurred predominantly in mature adipocytes, but not in the stromal vascular fraction in epididymal WAT and inguinal WAT (iWAT). Furthermore, a single bolus of recombinant mouse FGF21 injection increased VEGF levels in WAT. Long-term intermittent fasting for 16 weeks increased WAT angiogenesis, iWAT browning, and improved insulin resistance and inflammation, but the effect was blunted in FGF21 liver-specific knockout mice. In summary, these data
suggest that FGF21 is a potent regulator of VEGF levels in WAT. The interorgan FGF21 signaling-induced WAT angiogenesis by VEGF could be a potential new therapeutic target in combination with obesity-related metabolic disorders.

**Key Words:** fasting, FGF21, VEGF, angiogenesis, intermittent fasting

For centuries, food acquisition has become easier for humans, while obesity and related metabolic disorders, including type II diabetes, nonalcoholic fatty liver disease, cardiovascular disease, and cancer are becoming growing health challenges in modern society, which mainly result from a dysregulated balance between energy intake and energy expenditure (1). White adipose tissue (WAT), as the main organ storing excess energy, is essential for energy homeostasis and metabolic health. To support the metabolic change, the plasticity of the adipose tissues is crucial to maintain appropriate energy balance and secretion of endocrine factors (2,3). Caloric excess or high-fat diet (HFD) have been shown to suppress adipose angiogenesis, expand the lipid droplet size, and induce the infiltration of immune cells (4), which caused inflammation and insulin resistance. In contrast, fasting regimens or hypocaloric diets are associated with improved the metabolic and inflammatory diseases in humans (5). However, the exact mechanisms by which endocrine factor modulate adipose plasticity has not been well established, particularly the onset of adipose angiogenesis.

In contrast to WAT, brown adipose tissue (BAT) is a highly vascularized organ generating heat by metabolizing glucose and fatty acids (6,7). Emerging evidence has suggested that functional brown/beige fat may enhance energy expenditure via uncoupling protein 1 (UCP-1)–mediated thermogenesis (8). However, to support the supply of oxygen and nutrients to adipose tissue, the plasticity of the embedded vasculature in adipose tissues is crucial (2). Vascular endothelial growth factor (VEGF) is classically known to be essential in vascular development, which are activated upon stimuli such as exercise (9,10) and cold exposure (11,12). Recent studies have highlighted the critical role of adipose tissue VEGF in the control of adipose tissue metabolism and systemic energy homeostasis (13). Overexpression of VEGF in adipose tissue facilitates angiogenesis, increases inguinal WAT (iWAT) UCP-1 levels and resists HFD induced obesity (14,15). Furthermore, adipose-specific VEGF knockout or pharmacological VEGF blockade by antibodies abolished the browning of WAT (13). VEGF has also been reported to increased insulin sensitivity in genetic and HFD-fed obese mouse models (16). Taken together, these findings support the WAT VEGF as the critical factor of regulating angiogenesis and metabolic homeostasis.

Fibroblast growth factor 21 (FGF21) is an endocrine factor produced by several tissues, including the liver, adipose tissue, skeletal muscle, and heart, but the liver is the major source of FGF21 and contributes to approximately 90% of circulating FGF21 levels (17-21). The expression and secretion of hepatic FGF21 were reported to be affected by several nutritional factors (17,22-24), and fasting is one of the most important factors (25-27). Recent work has demonstrated that FGF21 stimulates thermogenic gene expression BAT and induces iWAT browning (20,28-30). In addition, FGF21 indirectly regulates BAT thermogenesis by acting on the central nervous system (31). However, the effect of FGF21 on the adipose angiogenesis remains to be investigated.

In the current study, we found that fasting induced a selective and drastic elevation in VEGF levels in WAT, but this did not occur in BAT and liver. Furthermore, the fasting-induced Vegfa expression occurred predominantly in mature adipocytes, but not in the stromal vascular fraction in epididymal WAT (eWAT) and iWAT. Long-term intermittent fasting (IF) increased WAT angiogenesis and iWAT browning and improved insulin resistance and inflammation, but was blunted in FGF21 liver-specific knockout (FGF21 LKO) mice. In summary, these data suggest that FGF21 is a potent regulator of VEGF levels in WAT and emphasize the link between the dietary patterns and adipose angiogenesis.

**Materials and Methods**

**Animals**

All animal procedures in this study were approved by the Institutional Animal Care and Research Committee of Sichuan Agricultural University (SICAU-2015-033). The mice were fed a standard diet (Figs. 1 and 2) comprising 70%, 20%, and 10% of calories from carbohydrate, protein, and fat, respectively (D12450J), or a HFD (Figs. 3 and 4) comprising 20%, 20%, and 60% of calories from carbohydrates, protein, and fat (D12492), from Research Diets (New Brunswick, NJ, USA). C57BL/6J male mice were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The FGF21 LKO mice were generated as previously described (20). FGF21Liver+/-, Alb-Cre mice were generated by mating FGF21-loxp/loxp mice (022361; The
Jackson Laboratory, Bar Harbor, ME, USA) with Alb-Cre mice (J003574; Model Animal Research Center, Nanjing University, Nanjing, China) transgenic mice. FGF21 Liver+/+, Alb-Cre mice were generated by crossing FGF21 Liver+/-, Alb-Cre mice with FGF21 loxp/loxp mice. Littermates of FGF21 loxp/loxp mice were used as control. Recombinant mouse FGF21 (8409; R&D Systems; Bio-Techne, Minneapolis, MN, USA) or equal volume of phosphate-buffered saline was injected from tail vein at the dose of 1 mg/kg bodyweight. Mice were kept in 24 ± 2°C facilities with a 12-h light/dark cycle and had free access to water.

Feeding schedule and diets

For IF, body weight-matched 8-week-old male C57BL/6J mice were either fed with chow diets ad libitum or fasting for various time periods (6 h, 12 h, and 24 h) as indicated. (A) Serum VEGF levels and (B) the mRNA expression of Vegfa in epididymal WAT (eWAT) (B), subcutaneous iWAT (C), interscapular BAT (D), liver (E), and muscle (F) as determined by real-time PCR analysis. The protein levels of VEGF in eWAT (G) and iWAT (H) as determined by western blot; (I) serum FGF21 levels as determined by enzyme-linked immunosorbent assay; and real-time transcription PCR analysis for Fgf21 mRNA expression levels of liver (J), eWAT (K), iWAT (L), BAT (M), and muscle (N). Serum FFA (O) and ketone bodies (P) levels. Data are mean ± SEM; n = 6/group. Statistical significance was evaluated by 1-way ANOVA with Tukey’s test for multiple comparisons to determine differences between each group. Labeled means without a common letter differ, \( P < 0.05 \).

Abbreviations: BAT, brown adipose tissue; eWAT, epididymal white adipose tissue; FFA, free fatty acid; FGF21, fibroblast growth factor 21; iWAT, inguinal white adipose tissue; VEGF, vascular endothelial growth factor.

Figure 1. Fasting induced adipose-specific VEGF expression and liver Fgf21 expression. Twelve-week-old male C57BL/6J mice were either fed with chow diets ad libitum or fasting for various time periods (6 h, 12 h, and 24 h) as indicated. (A) Serum VEGF levels and (B) the mRNA expression of Vegfa in epididymal WAT (eWAT) (B), subcutaneous iWAT (C), interscapular BAT (D), liver (E), and muscle (F) as determined by real-time PCR analysis. The protein levels of VEGF in eWAT (G) and iWAT (H) as determined by western blot; (I) serum FGF21 levels as determined by enzyme-linked immunosorbent assay; and real-time transcription PCR analysis for Fgf21 mRNA expression levels of liver (J), eWAT (K), iWAT (L), BAT (M), and muscle (N). Serum FFA (O) and ketone bodies (P) levels. Data are mean ± SEM; n = 6/group. Statistical significance was evaluated by 1-way ANOVA with Tukey’s test for multiple comparisons to determine differences between each group. Labeled means without a common letter differ, \( P < 0.05 \).
For one-time fasting, body weight-matched 12-week-old male C57BL/6J mice were fed with standard diet (D12450J), and the mice were killed at the same time (9:00 AM) after overnight fasting for 6 h, 12 h, and 24 h.

Metabolic profiling
Body composition was analyzed using the EchoMRI-100 machine (Echo Medical Systems, Houston, TX, USA), which determines fat and lean mass in conscious mice. Body metabolic rates were tested by indirect calorimetry in a comprehensive laboratory animal monitoring system (Columbus Instruments) for 2 days according to the manufacturer’s instructions. Light and feeding conditions were kept the same as in the regular cages.

Glucose tolerance test
Mice were fasted overnight. The next morning, mice fed with chow or a HFD were administered with D-glucose at the dose of 1 g/kg body weight, respectively, by intraperitoneal injection. Blood glucose levels were measured at 0, 15, 30, 45, 60, 90, and 120 min post injection with tail vein blood using glucose test strips (Roche Diagnostics).

Analysis of serum biochemistry and hormones
Mice were euthanized using carbon dioxide, followed by cervical dislocation. Serum was collected for further analysis. Serum free fatty acid and β-hydroxybutyrate levels were measured on an automatic biochemical analyzer (7020; Hitachi, Tokyo, Japan) with the respective analysis kits (Beijing Strong Biotechnologies, Beijing, China), according to the manufacturer’s instructions. Serum FGF21 (MF2100; R&D Systems; Bio-Techne, Minneapolis, MN, USA) and VEGF (KE10009; Proteintech Group, Rosemont, PA, USA) levels were measured with commercial enzyme-linked immunosorbent assay kits, according to the manufacturer’s instructions.

Isolation of adipocytes and stromal vascular cells from adipose tissue
Adipocytes and stromal vascular cells from adipose tissue were isolated as previously described (32). In brief, 1 eWAT or iWAT pad from 16-week-old male C57BL/6J mice was excised, weighed, and rinsed in isolation buffer. Fat pads were then cut into small pieces in isolation buffer supplemented with 1 mg/mL type I collagenase and digested at 37°C in shaking-water bath at 100 rpm for 45 min. Digested tissues were filtered through 400 mesh to get single-cell suspension. After centrifugation at 800 g, floating adipocytes and pellet cells were rinsed twice with isolation buffer. The adipocytes, pelleted stromal vascular cells, and the other fat pad were used for ribonucleic acid (RNA) extraction and determination of gene expression.
RNA extraction and gene expression analysis

RNA extraction and real-time polymerase chain reaction (PCR) were performed as previously reported (20). Briefly, RNA from adipose tissues were extracted by Trizol (15596018; Thermo Fisher Scientific) and purified using RNA mini-columns (RR037A; Takara Bio, Kusatsu, Japan). Reverse transcription and SYBR green quantitative PCR (RR820A; Takara Bio) were performed according to the manufacturer protocols. Target primer sequences are shown in Table 1.

Western blot analysis

Protein extraction and western blotting were performed as previously described (20). Sources of antibodies anti-VEGF (33) was purchased from Abcam (Cambridge, UK); anti-β-actin (34), from Cell Signaling Technology (Danvers, MA, USA); and UCP-1 (35), from Sigma-Aldrich.

Tissue histology and Immunohistochemistry

Adipose tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline and then dehydrated, embedded in paraffin, and sectioned (5-μm thickness). Sections were stained with hematoxylin and eosin or incubated with UCP-1 (Sigma-Aldrich) or CD31 antibody (36) followed by 3,3-diaminobenzidine staining and examined under bright-field microscopy (Nikon 80i). Representative of 3–4 biological replicates for histological analyses are shown.
Statistics
Data were analyzed by unpaired t test between 2 independent groups. To analyze more than 2 groups, 1-way analysis of variance (ANOVA) was used, and Tukey’s test for multiple comparisons to determine differences between each group with Prism 6 software (GraphPad Software, Table 1. List of primers used

| Name   | Forward          | Reverse           |
|--------|------------------|-------------------|
| β-actin| GCTGATTCCCCCTCCATCG | CCAGTTGTAACATGCCATGT |
| Ucp-1  | GCCAAAGTCGGCCTTACAT | CAGTTGCCAGCCTCCATGT |
| Fgf 21 | CTGCTGCGGCTACACCAAG | CTGCCTCTACGGCCTTAC |
| Pgc-1α | TATGGAGTGACATAGAGTGTGC | CTGCCTACGGCCTTAC |
| Dio2   | GCTTGCCAGGCTGCTTCTG | CGTGGCTGGAGGCTGCTT |
| Elovl6 | TCACCTTTCAGCATCACCT | CTGCTGAGGCTGCTT |
| Vegfa  | CTGTAACAGATGACGCTTGG | CTGCTGAAAGGCTGCTT |
| TNFα   | CAGGGTTCATGACGCTTGG | CTGCTGAGGCTGCTT |
| IL1β   | CTGGTGTGAGCCTTCCATT | CCGACACAGCAGGCTT |

Figure 4. Liver-FGF21 is required for intermittent fasting-induced metabolic benefits. Mice were fed with HFD ad libitum or time-restricted access to food for 16 weeks. (A) O₂ consumption (VO₂), (B) CO₂ production (VCO₂). (C and D) GTT shows normal glucose tolerance in HT mice but not for FGF21 LKO mice. (E) Serum insulin levels, real-time quantitative PCR analysis for mRNA expression levels of browning related genes (F), UCP-1 protein level (G) and immunohistochemical staining (H) of UCP-1 in iWAT. (I) The expression of pro-inflammatory cytokines (TNFα and IL1β) in eWAT. (J) A working model of dietary intake regulating iWAT browning via FGF21 signaling. Data are mean ± SEM; n = 6–8/group. Statistical significance was evaluated by the 2-way ANOVA test and the Tukey’s test for multiple comparisons to determine differences between each group. *P < 0.05, **P < 0.01; labeled means without a common letter differ, P < 0.05.
La Jolla, CA, USA). For the experiments involving FGF21 LKO mice, data were analyzed by the 2-way ANOVA test and the Tukey’s test for multiple comparisons to determine differences between each group. Data are presented as means ± standard error of mean (SEM). Statistical significance was set at P < 0.05.

Results
Liver FGF21 and adipose-specific VEGF expression are induced in parallel during fasting

To explore the physiological roles of FGF21 and VEGF in fasting, we first investigated the dynamic changes of FGF21 and VEGF expressions in various types of adipose tissues (iWAT, BAT, and eWAT), the liver, and the circulation levels at various time periods after fasting. Consistent with a previous report (37), there was a modest decline in serum level of VEGF after 6 h of fasting (Fig. 1A). Interestingly, Vegfa expression levels in eWAT (Fig. 1B) and iWAT (Fig. 1C) increased as early as 6 h of fasting. However, the Vegfa messenger (mRNA) expression levels in the BAT (Fig. 1D), liver (Fig. 1E), and muscle (Fig. 1F), remained unchanged. Consistently, VEGF protein levels were also increased in eWAT and iWAT by fasting (Fig. 1G and 1H). In agreement with previous reports (22,38), the increase of circulating FGF21 (Fig. 1I) and liver Fgf21 expression occurred as early as 6 h of fasting (Fig. 1J), while Fg21 expression in eWAT (Fig. 1K), iWAT (Fig. 1L), BAT (Fig. 1M), and muscle (Fig. 1N) remained unaltered. In addition, food removal for 12 h was sufficient to increase the free fatty acid (Fig. 1O) and ketone bodies levels (Fig. 1P). These results suggested that there may be a close relationship between liver FGF21 and adipose VEGF expression and fasting led to a selective upregulation of VEGF in WAT.

VEGF expression in adipocytes is induced by FGF21

We have showed that fasting dramatically induced VEGF expression in adipose tissue (Fig. 1G and 1H). To evaluate which kind of cells in WAT were involved in this process, primary adipocytes and stromal vascular cells were isolated from the iWAT and eWAT of fed or 24-h–fasted mice. Consistent with the previous results (Fig. 1G and 1H), fasting stimulated Vegfa expression in adipose tissues (Fig. 2A and 2B). Intriguingly, fasting only induced significant increase of Vegfa expression in adipocytes but not in stromal vascular cells neither in iWAT nor eWAT (Fig. 2A and 2B), indicating that adipocytes might be the producer of VEGF in response to fasting in vivo. To evaluate the effect of FGF21 on VEGF expression during fasting, wild-type and FGF21 LKO mice were fasting for 24 h. The mRNA levels of Vegfa in both iWAT and eWAT in wild-type mice were greater compared to FGF21 LKO mice after 24 h of fasting (Fig. 2C), which suggested that liver FGF21 play a role in fasted-induced WAT VEGF expression. To evaluate the direct effect of FGF21 on VEGF expression, mice were treated with a single bolus of recombinant mouse FGF21 (rmFGF21). Compared with the effects in control mice, rmFGF21 administration induced Vegfa mRNA expression levels after 3 h of rmFGF21 injection in both eWAT (Fig. 2D) and iWAT (Fig. 2E), but not in BAT (Fig. 2F). To our surprise, rmFGF21 injection increased the WAT VEGF expression levels as early as 1 h after injection (Fig. 2G and 2H), which suggested that FGF21 injection not only increase VEGF protein synthesis but also increase the protein stability.

Dietary intake induced WAT VEGF expression and angiogenesis requires liver FGF21 signaling

Liver-derived FGF21 is the main contributor to circulating FGF21 (20-22); thus, FGF21 LKO mice were used to examine the role of hepatic FGF21 in the regulation of WAT VEGF expression and angiogenesis induced by IF. Wild-type and FGF21 LKO mice on a HFD were fed ad libitum or subjected to IF for 16 weeks (Fig. 3A). Neither the genotype nor the feeding pattern affected food intake (data not shown). Intermittent fasting prevented body weight gain in WT mice but not in FGF21 LKO mice (Fig. 3B and 3C), which was accompanied by a reduction in fat composition in WT mice with time-restricted access to food (HT) and no effect was observed in FGF21 LKO mice (Fig. 3D). Since IF is a cyclic fast–refed pattern, serum and tissue samples were collected at 7:00 AM when the IF mice were fed for 10 h and at 9:00 PM when the IF mice were fasted for 14 h. Intermittent fasting increased serum FGF21 concentrations at both time points in WT mice, and the increase in serum FGF21 was especially augmented during fasting, which did not occur in FGF21 LKO mice (Fig. 3E). Intermittent fasting significantly increased WAT vascularization in WT mice, but not in FGF21 LKO mice (Fig. 3F). Indeed, IF induced VEGF mRNA and protein expression in iWAT and eWAT especially at night (Fig. 3G–I), which was also abolished in FGF21 LKO mice. Immunocytochemical analysis epitopes for the vasculatures, CD31, demonstrated that IF increased vascularization in WT mice, but not in FGF21 LKO mice (Fig. 3J). Collectively, these data strongly suggest that secretion of FGF21 is critical for IF on facilitating WAT VEGF expression and vascularization.
Liver FGF21 is required for dietary intake mediated metabolic benefits

To assess energy expenditure, a comprehensive laboratory animal monitoring system was used. The oxygen consumption (Fig. 4A) and carbon dioxide production (Fig. 4B) were not changed during the light cycle but were increased by IF in the WT mice only during the dark phase, but this effect was not observed in FGF21 LKO mice (Fig. 4A and 4B). The glucose tolerance test (Fig. 4C and 4D) and serum insulin levels (Fig. 4E) demonstrated that IF ameliorated HFD-induced insulin resistance in WT mice but not FGF21 LKO mice. FGF21 liver specific knock-out also abolished IF-induced expression of browning genes, such as Ucp-1, Pgc-1a, Dio2, and Elovl6 in iWAT both at 7:00 am and 9:00 pm (Fig. 4F).

Moreover, loss of liver FGF21 blocked the IF-driven induction of iWAT UCP-1 protein expression, as revealed by western blotting and immunohistochemistry analysis (Fig. 4G and 4H). Consistent with these changes, oxygen consumption (VO₂) remains unchanged, indicating that IF did not increased energy expenditure in FGF21 LKO mice (Fig. 4A). To explore whether IF was associated with changes in WAT inflammation, the inflammation related gene expression levels were performed. The expression of M1 macrophage polarization marker genes, including Tnfa, and IL1β, were decreased by IF in WT mice but not in FGF21 LKO mice (Fig. 4I). These data suggested that IF regulate the inflammation in WAT through FGF21 signaling. Collectively, these data suggested that enhanced secretion of FGF21 is critical for IF induced alleviation of the inflammation and HDF-induced insulin resistance.

Discussion

Obesity and the associated multiple comorbidities represent one of the most serious health issues affecting one third of global adults (39). Improvements in adipose tissue angiogenesis offer a potential therapeutic avenue for metabolic diseases (2). The present study showed that a drastic effect of 1-time and intermittent fasting on WAT angiogenesis, which promoted metabolic flexibility in adipose tissues and prevented obesity associated metabolic disorder. Furthermore, we found that fasting induced a selective and drastic elevation in VEGF levels in WAT via liver FGF21. The fasting-induced Vegfα expression occurred predominantly in mature adipocytes, but not in the stromal vascular fraction in eWAT and iWAT. Long-term IF increased WAT angiogenesis and iWAT browning, which improved insulin resistance via liver FGF21. In summary, these data suggest that FGF21 is a potent regulator of WAT VEGF levels and emphasize the link between the dietary patterns and adipose angiogenesis.

Adipose tissue is essential for energy homeostasis and metabolic health and is considered to be a main target for the treatment of obesity and diabetes (2,40). In the present study, based on the EchoMRT™ analysis showed IF specifically, reduced fat mass without any effect on lean body mass (Fig. 3D). To support the metabolic homeostasis, the plasticity of the adipose tissues is crucial for maintaining appropriate energy balance (2). Adipose tissues, especially BAT, had a high level of vessel density (6). Adipocytes have multiple functions that is required for energy homeostasis, such as oxygen and nutrient supply, and transports of metabolic factors. Adipocyte overexpressing VEGF triggered angiogenesis and contributed to resistance of HFD-induced obesity (13,14). The present work revealed that 1-time fasting and cyclical intermittent fasting induced a selective and drastic elevation of VEGF expression levels in WAT and increased the WAT angiogenesis and thermogenesis in intermittent fasting (Figs. 1G and H and 3F-J). In mammals, the liver is the peripheral integrator of nutrient availability and energy needs of the organism (41,42); therefore, liver-derived factors hold great promise as targets for WAT VEGF expression and obesity-related metabolic disorder.

Most researchers have focused on the role of a single tissue metabolism with particular interest in the adipose tissue to investigate the molecular mechanism of the beneficial effects of intermittent and periodic fasting on energy homeostasis (43-45). However, this feeding pattern induces a series of metabolic changes that might require interorgan crosstalk. In most of the mammalian species, the liver is the primary peripheral organ that senses circulating nutrient availability and is important for fasting, feeding, digestion, and metabolic balance (41,46). Liver-derived FGF21 is considered as a starvation hormone that could be rapidly induced in the liver by fasting (25,26), and, in turn, FGF21 acted as a critical endocrine signal to regulate the systemic energy metabolism (31,47). In the present study, liver Fgf21 expression and circulating FGF21 concentrations underwent coincident changes with WAT VEGF levels (Fig. 1G-I), confirming the close relationship between serum FGF21 levels and WAT VEGF expression. Interestingly, fasting increased VEGF expression in adipocytes but not in stromal vascular fraction in eWAT and iWAT (Fig. 2A and 2B), where there is high expression levels of FGF21 receptor Klothoβ and fibroblast growth factor receptor-1 (48). In the present study, we showed that a single bolus of recombinant mouse FGF21 increased VEGF levels in WAT (Fig. 2G and 2H). It is worth noting that FGF21 injection increased the VEGF protein expression before the changes of mRNA levels, which suggested that FGF21 not only increase VEGF protein synthesis but also increase the protein stability through unknown mechanisms.
The high thermogenic activity of adipose tissues requires a particularly high rate of blood perfusion both to supply O$_2$ and substrates and to export metabolic factors (2). VEGF has long been associated with increased vascular permeability and improves the access of metabolic substrates to tissues (49). VEGF is highly expressed in adipose tissue (6, 7) and overexpression of VEGF in adipocytes increases iWAT browning (4). In the present study, we showed that both acute fasting and IF induced substantial increase in VEGF expression in WAT. Enhanced oxygen consumption during the night in IF, which was consistent with the upregulated UCP-1 expression in iWAT. Periodic fasting resulted in benefits ranging from the prevention to the enhanced treatment of diseases (5,50). Consistent with previous investigations (5,43,45), the results of the present study revealed that IF significantly reduced HFD-induced body weight and adipose tissue gain, as well as improved glucose clearance (Fig. 3B-D and 4C-E). Recently, the beneficial effects of IF on adipose thermogenesis and M2 macrophage activation were reported to be dependent on adipose VEGF (37). Indeed, we found that both IF and acute fasting induced substantial increase in VEGF expression and the anti-inflammation related genes in adipose tissue in vivo (Fig. 3F-I and 4I). Interestingly, the effects of IF on VEGF expression and anti-inflammation were abolished when liver FGF21 was absent, suggested that the beneficial effects of IF on adipose tissues metabolism are mainly attributed to FGF21 signaling. In the present study, loss of liver FGF21 abolished the effects of IF on reducing body weight (Fig. 3B) and browning of adipose tissue (Fig. 4F), indicating the critical role of FGF21 in mediating liver-adipose crosstalk during IF. However, the body weight and adipose tissue weight of FGF21 LKO mice were greater than the WT mice in the HT group (Fig. 3B), and the reason for that need further investigation.

For the first time, our present study demonstrated that a drastic effect of 1-time and intermittent fasting induced a selective and drastic elevation in VEGF levels in WAT. In support of those findings, IF-induced WAT angiogenesis was abolished in FGF21 LKO mice during IF (Fig. 3F and 3J), suggesting that liver FGF21 is an important mediator in this process. However, it was unclear in the present study that the browning induced by FGF21 was attributed to its direct effect on adipocytes or its ability to induce VEGF-mediated browning. It will be interesting to examine the implication of FGF21-mediated metabolic benefits in this process. On the other hand, FGF21-induced VEGF expression may improve adipocyte access to metabolic substrates, which, in turn, promotes beige adipocyte development.

In conclusion, the present study showed a drastic effect of intermittent fasting on WAT angiogenesis, which promoted the metabolic flexibility of adipose tissues and prevented obesity-associated metabolic disorder (Fig. 4J). Furthermore, we found that fasting induced a selective and drastic elevation in VEGF levels in WAT. Long-term IF regulated WAT angiogenesis, iWAT browning, insulin resistance, and inflammation via liver FGF21. Based on the results in the present study, the interorgan FGF21 signaling induced WAT angiogenesis by VEGF could be a novel potential therapeutic target in combination with obesity-related metabolic disorders.

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Data Availability: Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References. The data that support the findings of this study are available from De Wu or Yong Zhuo upon reasonable request.

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