Brief Communication

MOLECULAR METABOLISM

Depot-specific differences in angiogenic capacity of adipose tissue in differential susceptibility to diet-induced obesity

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ABSTRACT

Objective: Adipose tissue (AT) expansion requires AT remodeling, which depends on AT angiogenesis. Modulation of AT angiogenesis could have therapeutic promise for the treatment of obesity. However, it is unclear how the capacity of angiogenesis in each adipose depot is affected by over-nutrition. Therefore, we investigated the angiogenic capacity (AC) of subcutaneous and visceral fats in lean and obese mice.
Methods: We compared the AC of epididymal fat (EF) and inguinal fat (IF) using an angiogenesis assay in diet-induced obese (DIO) mice and diet-resistant (DR) mice fed a high-fat diet (HFD). Furthermore, we compared the expression levels of genes related to angiogenesis, macrophage recruitment, and inflammation using RT-qPCR in the EF and IF of lean mice fed a low-fat diet (LFD), DIO mice, and DR mice fed a HFD.
Results: DIO mice showed a significant increase in the AC of EF only at 22 weeks of age compared to DR mice. The expression levels of genes related to angiogenesis, macrophage recruitment, and inflammation were significantly higher in the EF of DIO mice than in those of LFD mice and DR mice, while expression levels of genes related to macrophages and their recruitment were higher in the IF of DIO mice than in those of LFD and DR mice. Expression of genes related to angiogenesis (including *Hif1a*, *Vegfa*, *Fgf1*, *Kdr*, and *Pecam1*), macrophage recruitment, and inflammation (including *Emr1*, *Ccr2*, *Itgax*, *Ccl2*, *Tnf*, and *Il1b*) correlated more strongly with body weight in the EF of HFD-fed obese mice compared to that of IF.
Conclusions: These results suggest depot-specific differences in AT angiogenesis and a potential role in the susceptibility to diet-induced obesity.

Keywords Angiogenesis; Inflammation; Adipose tissue; Diet-induced obese mice; Diet-resistant mice; High-fat diet

1. INTRODUCTION

Obesity is a chronic disease that can cause diabetes and cardiovascular disease. To suppress its rapidly growing prevalence, several treatments have been suggested, including the modulation of angiogenesis in adipose tissue (AT). AT expansion is highly dependent on its angiogenesis, similar to tumor growth. Inhibition of angiogenesis in white adipose tissue can successfully reduce body weight and fat mass, as well as rapidly restore impaired glucose regulation in obese mice [1–4].

Hypoxia has been observed in the AT of obese humans and animals, but not in other tissues, including liver and skeletal muscle [5–7]. AT vascular density is defined as the number of vessels per adipocyte, which is decreased in obesity, suggesting an insufficient vascularization in AT due to its demand [8–11]. Therefore, AT hypoxia may contribute to increased angiogenesis in obesity. AT angiogenesis may be altered, depending on obesity status [8,12,13]. In addition, the levels of AT vascular endothelial growth factor (VEGF) expression, a key pro-angiogenic factor, vary in obesity, while VEGF plasma levels are increased in obese animals and humans [5,9,11,14]. Furthermore, the role of AT VEGF signaling in the development of obesity and insulin resistance may differ depending on obesity status [15]. Inadequate AT VEGF signaling could be a critical contributing factor to metabolic disturbance [16]. Therefore, AT vasculature may play a diverse role in obesity.

AT characteristics are depot-specific, and each depot is differentially associated with insulin resistance. Visceral fat is more highly correlated with insulin resistance and contains more inflammatory cells and smaller, more insulin-resistant and lipolytic adipocytes compared to subcutaneous fat in obese humans and rodents [17,18]. However, the

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depot specificity of AT angiogenesis remains unclear, as fat depot-specific differences in the degree of angiogenic capacity have varied in human studies [8,12,18]. Therefore, the depot specificity of AT angiogenic capacity needs to be determined.

Here, we investigated whether angiogenic capacity is depot-specific by analyzing angiogenic sprouting of epididymal and inguinal fats (EF and IF, respectively) from diet-induced obese (DIO) mice and diet-resistant (DR) mice fed a high-fat diet (HFD). In addition, we compared angiogenic capacity by analyzing the expression levels of angiogenic genes along with macrophages and inflammation in the AT of lean mice on a low-fat diet (LFD), DIO mice, and DR mice on a HFD. Furthermore, we compared the correlations between body weight and angiogenic gene expression levels, macrophages, and inflammation in visceral EF and subcutaneous IF in mice fed LFD and HFD.

2. MATERIALS AND METHODS

2.1. Animals

Male C57BL/6 mice (n = 8; Orient Bio Inc., Seoul, Korea) were used to compare the angiogenic capacity of epididymal fat in lean and obese mice at 24-weeks-old, as assessed by an angiogenesis assay. They were fed chow (5L79, Orient Bio Inc., Korea) or a high-fat diet (HFD; 45% calories as fat containing 20% protein, 35% carbohydrate; D12451; Research Diets, New Brunswick, NJ) *ad libitum* for 16 weeks until sacrifice.

Male C57BL/6 mice (n = 40; Central Lab Animal Inc., Seoul, Korea) were fed a HFD *ad libitum* for 14 weeks. The mice whose body weight were in the upper and lower 20% after 14 weeks of HFD were designated as DIO mice and DR mice, respectively. DIO mice and DR mice were used to compare the angiogenic capacity between EF and IF at 22-weeks-old, as assessed by an angiogenesis assay.

In a separate experiment, AT angiogenic capacity and inflammation were compared in lean mice fed a LFD (10% calories from fat, containing 20% protein, 35% carbohydrate 70%; D12450B; Research Diets) and in DIO mice and DR mice on the HFD (n = 40; male 8-week-old C57BL/6 mice, Central Lab Animal Inc., Seoul, Korea). Mice were divided into 2 groups and fed LFD or HFD for 12 weeks. The mice that met the criteria as DIO and DR mice were selected respectively.

Mice were maintained in a temperature- and humidity-controlled room (22 °C, 50% relative humidity) and were housed individually in standard mouse cages under a 12-h light/dark cycle. Food and water were provided *ad libitum*. All experimental procedures were performed according to the guidelines on the ethical use of animals issued by the Animal Care and Use Committee of Korea University and were approved by the Institutional Animal Care & Use Committee of Korea University.

2.2. Body weight, food intake and body composition

Body weight and food intake were monitored once a week throughout the experiment. Body composition was measured with a mini-spec nuclear magnetic resonance spectrometer (Bruker Corporation, Seoul, Korea).

2.3. Angiogenesis analysis of adipose tissue

To compare fat depot-specific differences in angiogenic capacity, we previously modified an angiogenesis assay for adipose tissue [13]. Briefly, EF and IF of C57BL/6 mice were freshly obtained and placed in a petri dish containing EGM-2 MV-supplemented EBM-2 medium (Lonza, Basel, Switzerland).

Adipose tissue was minced into <1 mm^3 pieces under sterile conditions and digested in a tube containing 1 mg/ml of collagenase type I (Sigma-Aldrich Co., St. Louis, Missouri, United States) for 30 min at 37 °C in a shaking water bath. After enzymatic digestion, tissue pieces were washed with EBM-2 (Lonza, Basel, Switzerland) and filtered through a 100-μm cell strainer (BD, Franklin Lakes, New Jersey, USA). The unfiltered pieces with both stromal vascular fractions and a small number of adipocytes were collected with a needle and placed in each well of a 96-well plate containing Matrigel (BD, Franklin Lakes, New Jersey, USA). After embedding, explants were covered with Matrigel and incubated in a CO_2 incubator at 37 °C for 20 min. EGM-2 MV medium was added into each well, with half of the medium exchanged every other day.

Growing sprouts from EF and IF explants were observed under 100× magnification. The number of generated angiogenic sprouts was quantified by counting the points where at least three angiogenic branches met together.

Table 1 — Correlation coefficient between body weight and relative expression levels of genes involved in angiogenesis and inflammation in adipose tissue of mice fed a high-fat diet for 12 weeks. An asterisk indicates a significant difference in the slope of the least square line between epididymal and inguinal fat, both of which were significant ($P < 0.05$).

	Epididymal fat		Inguinal fat	
	r	P-value	r	P-value
Vegfa	0.517	0.002	0.040	0.824
*Fgf1**	0.515	0.003	0.417	0.018
Fgf2	0.221	0.218	0.157	0.382
*Pecam1**	0.697	<0.001	0.428	0.013
*Kdr**	0.483	0.004	0.362	0.038
*Hif1a**	0.670	<0.001	0.386	0.026
*Emr1**	0.783	<0.001	0.622	<0.001
Itgax	0.729	<0.001	0.173	0.454
Ccl2	0.810	<0.001	0.341	0.052
*Ccr2**	0.735	<0.001	0.495	0.003
Il6	0.313	0.077	0.520	0.002
Tnf-α	0.697	<0.001	0.088	0.746
Il1b	0.575	<0.001	0.056	0.836

2.4. Quantitative real-time PCR analysis

Mice were sacrificed after a 5-h fast. The epididymal and inguinal fats were dissected and frozen in 2-methylbutane on dry ice. RNA extraction was performed using TRIZOL (Thermo Fisher Scientific Waltham, Massachusetts, USA). The extracted RNA was measured by NanoDrop 2000c (Thermo Fisher Scientific Waltham, Massachusetts, USA). cDNA was synthesized using 1 μg of total RNA and an iScript cDNA synthesis kit (BioRad, Hercules, California, USA).

For quantitative real-time PCR (RT-qPCR), the TaqMan RT-PCR Ready-Mix Kit (PE Applied Biosystems, Waltham, Massachusetts, USA) and an ABI 7500 (Applied Biosystems, Waltham, Massachusetts, USA) were used. Forty PCR cycles were accomplished with a 2-step amplification (95 °C for 10 s, annealing temperature of 60 °C for 30 s) using the 7500 software (v2.0.6, Applied Biosystems, USA). Mouse primers are listed in Supplemental Table 1. Relative gene expression was normalized to Ribosomal protein L32 (*Rpl32*), a housekeeping gene, by subtracting the CT of *Rpl32* from that of the gene of interest. For the comparison of gene expression in AT of DIO and DR mice, the ΔΔCT was used to calculate and compare the approximate fold difference [19]. The correlation between body weight and the gene expression of interest was analyzed using the $2^{-\Delta\Delta CT}$ method [20].

2.5. Statistics

All results are presented as mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism 6.0 software (GraphPad Software

Inc., San Diego, CA). Angiogenic capacity differences assessed by an angiogenesis assay were analyzed by a Student's *t*-test.

Differences in body weight, cumulative energy intake, and body composition were analyzed by one-way ANOVA followed by a Tukey's multiple comparison test. Depot-specific differences in the angiogenic capacity-related gene expression and inflammation in DIO and DR mice were analyzed by Student's *t*-test. The correlations between body weight and gene expression were analyzed by a two-tailed Pearson's correlation and presented as a scatterplot and Pearson's correlation coefficient. To compare the slope of least square line, the method equivalent to an Analysis of Covariance (ANCOVA) was used. Statistical significance was set at a P-value <0.05 (Table 1 and Supplemental Table 2).

3. RESULTS

3.1. Changes in body weight, cumulative food intake, and body composition in lean mice on a low-fat diet, diet-induced obese mice and diet-resistant mice on a high-fat diet

The body weight and weight gain of DIO mice were significantly higher than those of DR mice and LFD mice after 12 weeks of HFD or LFD feeding. No significant differences were observed between DR and LFD mice (Figure 1A,C). Cumulative energy intake of DIO mice was significantly greater than that of DR and LFD mice, and that of DR mice was significantly greater than that of LFD mice (Figure 1B).

Fat and lean masses of DIO mice were significantly greater than those of DR and LFD mice, while no significant differences were observed between DR and LFD mice (Figure 1D,E). Both fat and lean masses were strongly correlated with body weight ($r = 0.974$ and $r = 0.747$; both $P < 0.05$).

3.2. Effect of susceptibility to diet-induced obesity on angiogenic capacity of epididymal fat in diet-induced obese and diet-resistant mice

To investigate the effect of HFD feeding on the angiogenic capacity of visceral fat, we performed an angiogenesis assay on EF in age-matched lean and obese mice fed either chow or a HFD at 24 weeks of age. Sixteen weeks of HFD increased the angiogenic sprouting compared to the same exposure of chow (Supplemental Figure 1).

Next, we performed an angiogenesis assay on EF in age-matched DIO and DR mice fed a HFD at 22 weeks of age to investigate the effect of susceptibility to diet-induced obesity on the angiogenic capacity of visceral fat. HFD was given for 14 weeks to old mice from 8 weeks of age. The number of angiogenic sprouting events of epididymal fat was greater in DIO mice compared to DR mice on the same diet ($P < 0.05$; Figure 2A).

To further investigate whether angiogenic capacity was altered in visceral fat between LFD, DIO, and DR mice, we measured the levels of gene expression related to angiogenesis in EF. *Hif1a* expression levels were significantly higher in EF of DIO mice than in that of DR and LFD mice. Expression levels of pro-angiogenesis-related genes, including *Vegfa*, *Kdr*, and *Fgf1*, were significantly higher in EF of DIO mice than in that of DR and LFD mice. Additionally, the *Pecam1* expression levels were higher in the EF of DIO mice by 20-fold than in that of DR and LFD mice (Figure 2B).

3.3. Effect of susceptibility to diet-induced obesity on angiogenic capacity of inguinal fat in diet-induced obese and diet-resistant mice

To investigate the effects of susceptibility to diet-induced obesity on the angiogenic capacity of subcutaneous fat, IF was analyzed from the same mice as in the EF study. No significant difference in the number of angiogenic sprouting events was observed for the IF between DIO mice and DR mice (Figure 3A).

To further investigate the angiogenic capacity of subcutaneous fat, we compared the expression of genes related to angiogenesis in IF of LFD, DIO, and DR mice. *Hif1a* expression levels were not significantly different in IF among the groups. *Pecam1* and *Kdr* gene expression levels were significantly higher in IF of DIO mice than in that of DR mice, with no significant change in *Fgf1* and *Fgf2* expression between the groups. Interestingly, *Vegfa* expression levels were significantly lower in DIO and DR mice than in LFD mice, with no significant differences between DIO and DR mice. In addition, *Kdr* expression levels were significantly lower in DR mice than in LFD mice, indicating a potential role for angiogenesis of IF in diet resistance (Figure 3B).

3.4. Differences in expression levels of genes related to inflammation in visceral and subcutaneous fat in lean mice on a low-fat diet, diet-induced obese mice, and diet-resistant mice on a high-fat diet

Expression levels of genes related to macrophages and their recruitment, including *Emr1*, *Itgax*, *Ccl2*, and *Ccr2*, as well as of pro-inflammation genes, including *Il6*, *Tnf*, and *Il1b*, were significantly higher in EF of DIO mice than in that of DR or LFD mice (Figure 2C,D).

Similarly, expression levels related to macrophages and their recruitment, including *Emr1*, *Ccl2*, and *Ccr2*, were significantly higher in IF of DIO mice than in that of DR and LFD mice. However, expression levels of pro-inflammation genes, including *Il6*, *Tnf*, and *Il1b*, were not significantly different between these groups (Figure 3C,D).

3.5. Depot-specific differences in the correlation of angiogenic capacity and inflammation with body weight and fat mass in mice on a low-fat and high-fat diet

We observed depot-specific differences in expression levels of genes related to angiogenesis and inflammation between EF and IF of LFD mice. *Hif1a*, *Vegfa*, *Fgf1*, *Fgf2*, and *Kdr* were highly expressed in EF compared to IF in LFD mice. However, *Pecam1* expression levels were significantly lower in EF compared to IF in LFD mice (Supplemental Figure 2A). Genes related to macrophage recruitment, including *Emr1*, *Ccr2*, and *Ccl2*, were highly expressed in EF compared to IF in LFD mice, whereas *Il6* expression levels were significantly lower in LFD mice (Supplemental Figure 2B and 2C).

To investigate whether changes in body weight and fat mass were associated with angiogenic capacity and inflammation in a depot-dependent manner after HFD feeding, we compared correlations between body weight, fat mass, and expression levels of genes related to angiogenesis and inflammation in both EF and IF in mice on the HFD.

Genes involved in angiogenesis and inflammation, except *Pecam1* and *Il6*, were expressed at higher levels in EF compared to IF (Figure 4, Supplemental Figure 3; Table 1). Expression levels of all genes, except *Fgf2* and *Il6*, were significantly correlated with body weight in EF ($P < 0.05$), whereas gene expression of *Vegfa*, *Fgf2*, *Itgax*, *Ccl2*, *Tnf*, and *Il1b* were not correlated in IF (Figure 4, Supplemental Figure 3; Table 1).

Moreover, correlations between these genes (except *Il6*) and body weight were stronger in EF than IF (Table 1). Gene expression levels, including *Vegfa*, *Itgax*, *Ccl2*, *Tnf*, and *Il1b*, were only significantly correlated with body weight in EF. However, *Il6* expression levels were significantly correlated with body weight in IF only. The slope of the

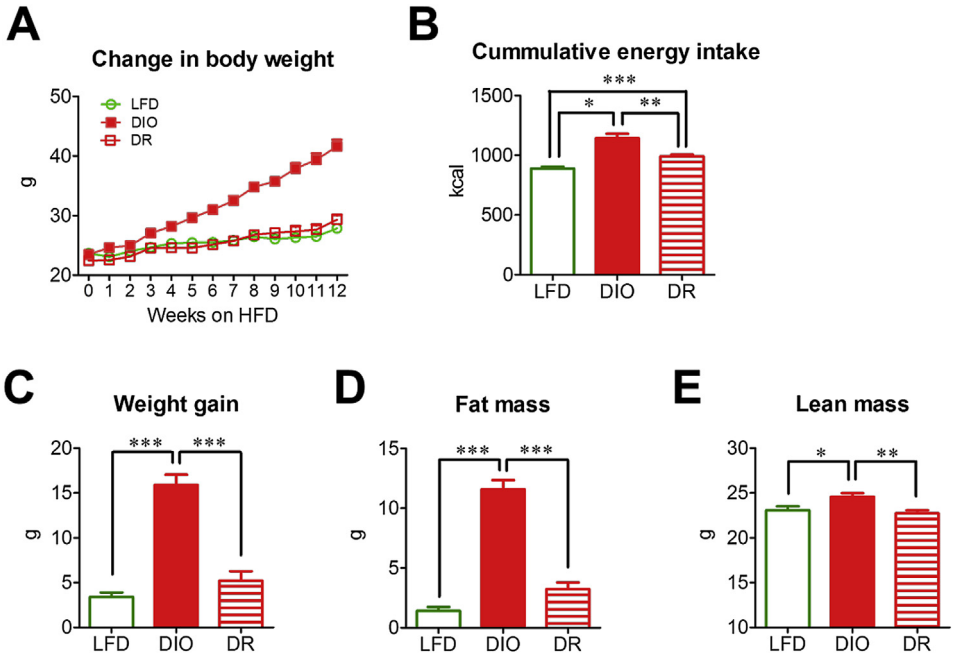

Figure 1: Changes in body weight (A), cumulative energy intake (B), weight gain (C), and body composition (D and E) of lean mice on a low-fat diet (LFD; n = 8), diet-induced obese mice (DIO; n = 6), and diet-resistant mice (DR; n = 6) on a high-fat diet (HFD). *P < 0.05; **P < 0.01; ***P < 0.001.

least square line was significantly different in *Hif1a*, *Fgf1*, *Kdr*, *Pecam1*, *Emr1*, and *Ccr2*, when comparing genes which had significant correlation in both EF and IF (all, P < 0.01; Figure 4, Supplemental Figure 3, and Table 1).

Similar to the correlations of the genes with body weight, expression levels of genes related to angiogenesis and inflammation were correlated with fat mass in both EF and IF (P < 0.05; Supplemental Figures 4 and 5, and Supplemental Table 2).

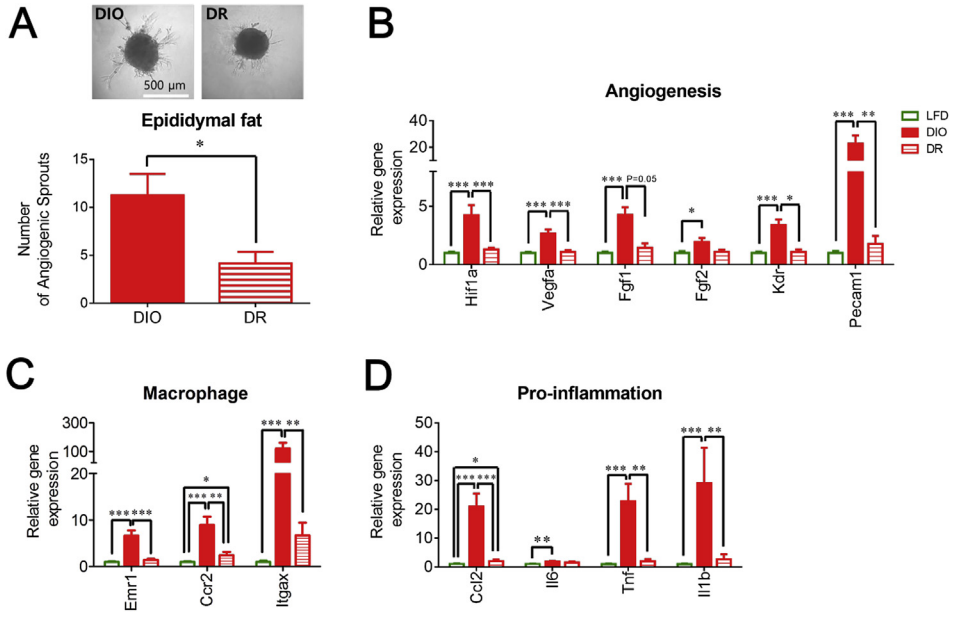

Figure 2: Comparison of angiogenic sprouting from explants of epididymal fat (A) in diet-induced obese (DIO; n = 6) and diet-resistant (DR; n = 6) mice on a high-fat diet (HFD) at 22 weeks of age. Representative figures of angiogenic sprouting of epididymal fat in DIO and DR mice (upper panel). The number of angiogenic sproutings of epididymal fat (lower panel). The number of angiogenic sproutings was measured on Day 4 after implantation of adipose tissue explants from epididymal fat. Comparison of relative expression levels of genes related to angiogenesis (B), macrophage recruitment (C), and pro-inflammation (D) in epididymal fat of lean mice on a low-fat diet (LFD; n = 8), DIO mice (n = 6) and DR mice (n = 6) on a HFD at 20-weeks-old. *P < 0.05; **P < 0.01; ***P < 0.001. *Ccl2*, chemokine (C-C motif) ligand 2; *Ccr2*, chemokine (C-C motif) receptor 2; *Emr1*, egf-like module containing, mucin-like, hormone receptor-like 1; *Fgf1*, fibroblast growth factor 1; *Fgf2*, fibroblast growth factor 2; *Hif1a*, hypoxia inducible factor 1, alpha subunit; *Il1b*, interleukin 1, beta; *Il6*, interleukin-6; *Itgax*, integrin, alpha X (complement component 3 receptor 4 subunit); *Kdr*, kinase insert domain protein receptor; *Pecam1*, platelet/endothelial cell adhesion molecule 1; *Tnf*, tumor necrosis factor; *Vegfa*, vascular endothelial growth factor A.

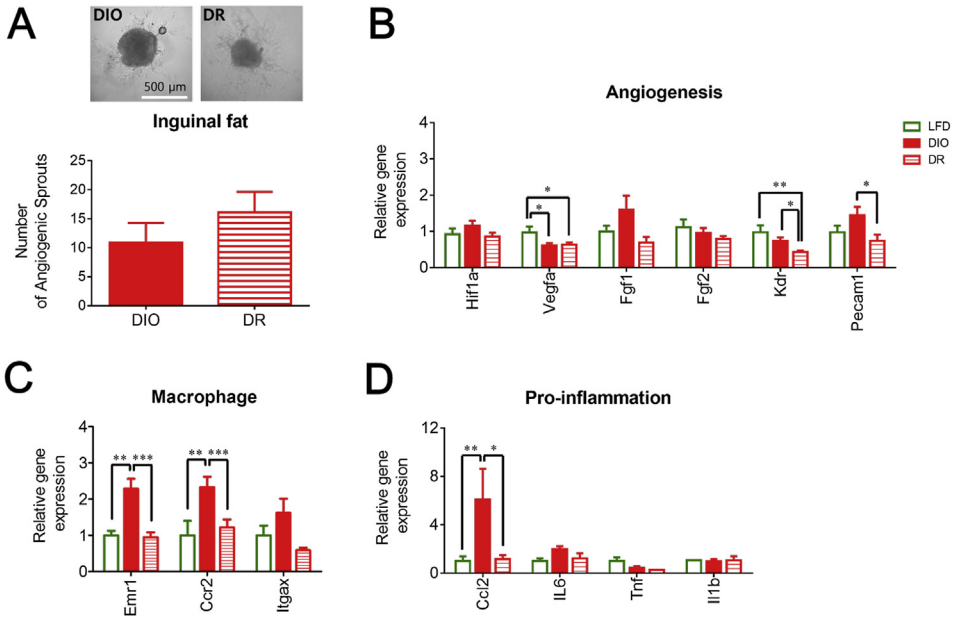

Figure 3: Comparison of angiogenic sprouting from explants of inguinal fat (A) in diet-induced obese (DIO; n = 6) and diet-resistant (DR; n = 6) mice on a HFD at 22 weeks of age. Representative figures of angiogenic sprouting of inguinal fat in DIO and DR mice (upper panel). The number of angiogenic sproutings of inguinal fat (lower panel). The number of angiogenic sproutings was measured on Day 4 after implantation of adipose tissue explants from inguinal fat. Comparison of relative expression levels of genes related to angiogenesis (B), macrophage recruitment (C), and pro-inflammation (D) in inguinal fat of lean mice on a low-fat diet (LFD n = 8), DIO mice (n = 6) and DR mice (n = 6) on a high-fat diet (HFD) at 20-weeks-old. *P < 0.05; **P < 0.01; ***P < 0.001. *Ccl2*, chemokine (C-C motif) ligand 2; *Ccr2*, chemokine (C-C motif) receptor 2; *Emr1*, egf-like module containing, mucin-like, hormone receptor-like 1; *Fgf1*, fibroblast growth factor 1; *Fgf2*, fibroblast growth factor 2; *Hif1a*, hypoxia inducible factor 1, alpha subunit; *Il1b*, interleukin 1, beta; *Il6*, interleukin-6; *Itgax*, integrin, alpha X (complement component 3 receptor 4 subunit); *Kdr*, kinase insert domain protein receptor; *Pecam1*, platelet/endothelial cell adhesion molecule 1; *Tnf*, tumor necrosis factor; *Vegfa*, vascular endothelial growth factor A.

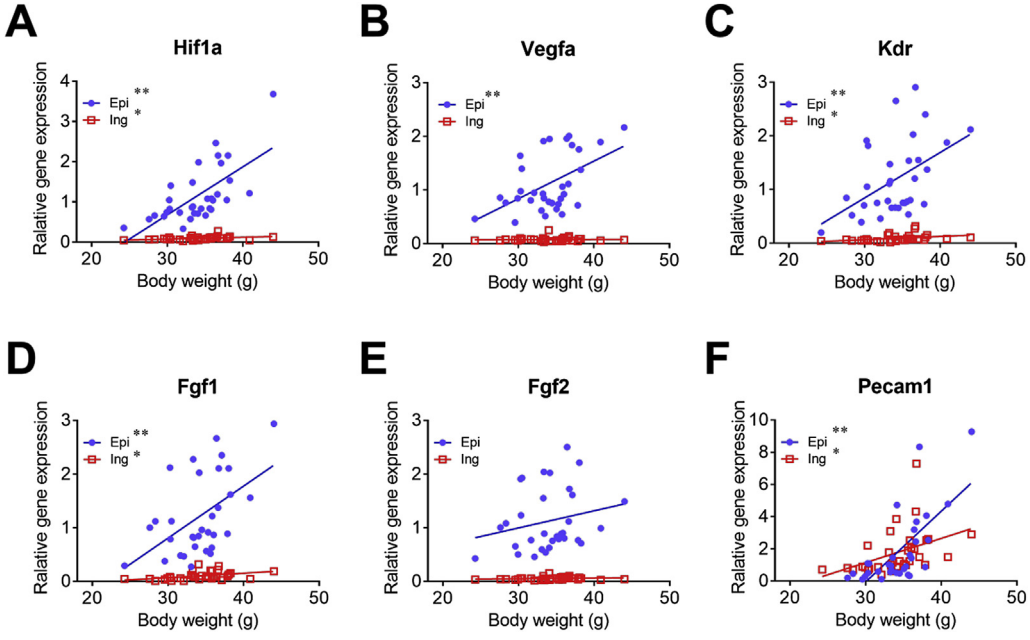

Figure 4: Correlation of body weight with expression levels of genes related to angiogenesis in epididymal fat and inguinal fat. Asterisks (*) indicate a significant correlation between expression of the gene of interest and body weight. *P < 0.05; **P < 0.01. *Fgf1*, fibroblast growth factor 1; *Fgf2*, fibroblast growth factor 2; *Hif1a*, hypoxia inducible factor 1, alpha subunit; *Kdr*, kinase insert domain protein receptor; *Pecam1*, platelet/endothelial cell adhesion molecule 1; *Vegfa*, vascular endothelial growth factor A.

4. DISCUSSION

We sought to clarify the differences in angiogenic capacity between subcutaneous and visceral fats in lean and obese mice. Adipose tissue is depot-specific for various characteristics including lipolysis, innervation, and adipokines [17]. It is important to identify differences between fats to better understand the role of adipose tissue in metabolism. However, angiogenesis in each depot is varied and needs

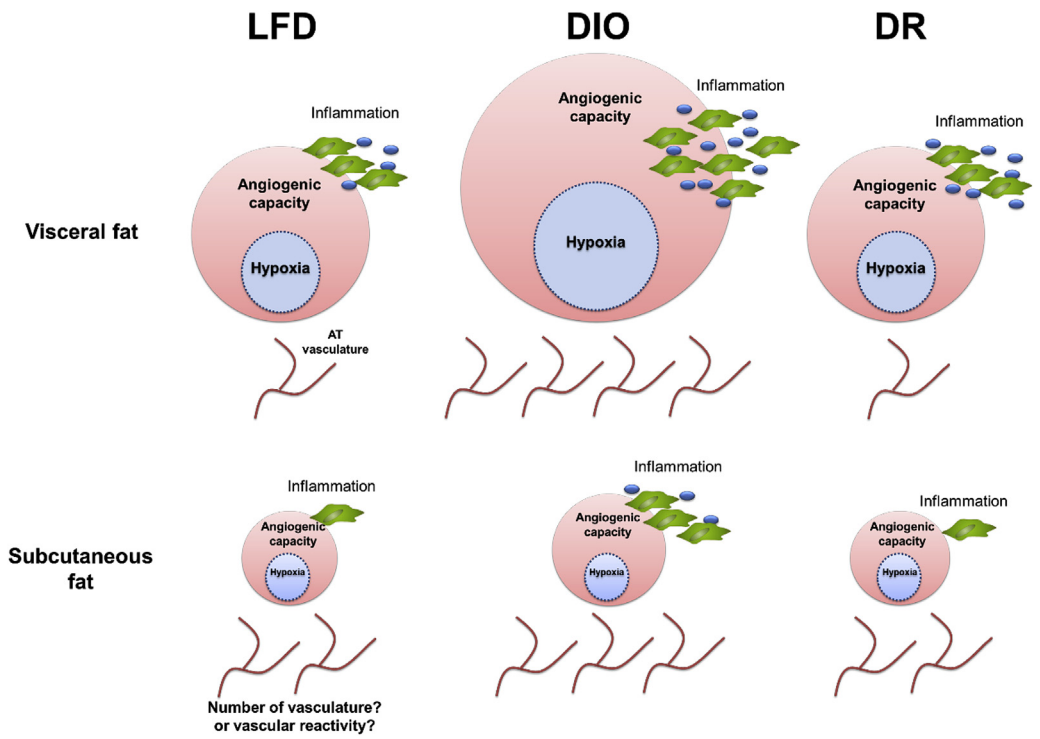

Figure 5: Schematic diagram of the difference in angiogenic capacity between epididymal fat and inguinal fat. Angiogenic capacity along with inflammation was greater in epididymal fat of lean mice fed a low-fat diet (LFD) relative to inguinal fat. The greater angiogenic capacity of epididymal fat relative to inguinal fat may be attributable to increased adipose tissue hypoxia, probably due to insufficient vasculature or vascular function to support adipocytes. High-fat diet (HFD) feeding potentiated angiogenic capacity and inflammation in epididymal fat of diet-induced obese (DIO) mice, while the effect was much lower in inguinal fat. Angiogenic capacity and inflammation of both fats was lower in diet-resistant (DR) mice relative to DIO mice, but those were similar to LFD mice. This study showed the differential ability of angiogenesis and responses to HFD in each depot.

to be determined [8,12,18]. Here, we showed that visceral EF had more angiogenic capacity than subcutaneous IF in obese mice using an angiogenesis assay and comparing angiogenic gene expression levels. *Vegfa* and its receptor, *Kdr*, are key pro-angiogenic factors for adipose tissue expansion [15,21,22]. These gene expression patterns were different between epididymal and inguinal fats in obese mice. *Vegfa* expression levels in EF were proportionate to body weight and fat mass increases in obese mice on a HFD. However, this relationship was not detected in IF of obese mice on the HFD. Surprisingly, *Vegfa* expression levels were significantly lower in IF of obese mice on the HFD relative to those of lean mice on a LFD, indicating a differential ability of angiogenesis in EF. *Kdr*, a VEGF receptor 2, is a critical receptor for modulating adipose tissue angiogenesis [22]. Similar to *Vegfa* expression, *Kdr* expression levels were lower in mice on the HFD relative to mice on the LFD (Figure 3). HFD feeding may differentially affect VEGF signaling in each depot, or differential demand for vascularization may exist, depending on fat localization.

Adipose tissue hypoxia occurs in obese humans and animals [5–7,14], and hypoxia-induced increases in Hif-1alpha may be a key factor to initiate AT angiogenesis [14]. Here, *Hif1a* expression patterns were different between EF and IF in obese mice. In EF of obese mice, *Hif1a* expression levels increased with increases in body weight and fat mass; this was higher in DIO mice relative to those of lean mice on either HFD or LFD. However, this was not observed in IF. Along with *Vegfa* and *Kdr* expression levels, these data strongly support the greater angiogenic capacity of EF, presumably due to the higher level of AT hypoxia relative to IF.

Next, we compared *Pecam1* expression levels, an endothelial marker, in both types of fat of lean and obese mice. Previously, *Pecam1* expression and protein levels were lower in AT of humans and rodents with obesity, which may represent AT vascular density [8,10,12,13]. Unexpectedly, *Pecam1* expression levels were significantly correlated with body weight and fat mass in both EF and IF of obese mice. This correlation was stronger in EF in mice on the HFD relative to IF. Interestingly, the *Pecam1* expression levels were significantly lower in EF of LFD mice relative to IF. Given the higher expression of *Hif1a*, *Vegfa*, *Fgf1*, *Fgf2*, and *Kdr* in EF of LFD mice, the lower expression levels of *Pecam1* in EF may indicate insufficient vasculature for the demand in EF relative to IF ($P < 0.05$; Supplemental Figure 2). However, after 12 weeks of HFD, *Pecam1* expression levels were greater in EF relative to inguinal fat. Therefore, angiogenic capacity may be greater in EF of LFD mice, possibly due to increased hypoxia relative to IF. This may be induced by insufficient vasculature or lower vascular reactivity. HFD feeding may greatly potentiate angiogenesis in EF relative to IF (Figure 5).

To compare the angiogenic capacity of both types of fat in lean and obese mice directly, we performed an angiogenesis assay in age-matched mice fed the HFD. Angiogenesis is determined by the net balance between pro-angiogenesis and anti-angiogenesis. Neo-angiogenesis consists of continual multiple steps including endothelium activation, proliferation, migration, and stabilization of newly-formed vasculature [23]. Adipose angiogenic capacity has been evaluated in previous studies [13,18], which has suggested a clear difference in angiogenic capacity between AT and other vascular tissue in obesity [13]. In a study using direct incubation of fats on chick chorioallantoic membrane, there was no difference in angiogenic capacity between subcutaneous fat and visceral fat in humans with obesity [18]. However, in a study using an ex vivo angiogenesis

assay of AT explants, angiogenic capacity of subcutaneous fat decreased as body weight increased, with no correlation between visceral fat of humans and obesity [8]. In contrast to human studies, angiogenic capacity increased in visceral fat of obese mice compared to lean mice on the different diets [13]. Therefore, it was necessary to clarify the effect of body status on angiogenic capacity in each depot. In ex vivo angiogenesis assay studies, we observed that there were significant increases in angiogenic capacity of visceral fat in DIO mice compared to DR mice (Figure 2) and in mice on a HFD compared to chow diet at the same age (Supplemental Figure 1). Long-term HFD feeding potentiated the angiogenic sprouting in EF of mice more susceptible to HFD. However, this angiogenic capacity response was much lower in IF relative to EF. These results strongly supported the hypothesis that long-term HFD exposure greatly increased angiogenic capacity of visceral EF in obese mice compared to subcutaneous IF. Given the discrepancy in angiogenic capacity between human and animal studies, it emphasized that more attention should be paid in application of the results from animal studies to clinical studies or more factors including sex and age may be considered in human studies regarding AT angiogenesis.

AT angiogenesis is closely associated with AT inflammation, and it has been hypothesized that AT hypoxia triggers angiogenesis and inflammation for AT remodeling in obesity [10,24]. The significant role of AT inflammation has been suggested in the development of obesity and insulin resistance [24,25]. Therefore, we investigated the status of macrophages and inflammation in both fats of lean and obese mice. Consistent with previous studies [25–27], *Emr1* (F4/80) and *Ccr2* expression levels were positively correlated with body weight and fat mass in both EF and IF of obese mice on the HFD (Table 1; Supplemental Table 2). However, we found that expression levels of *Itgax*, a macrophage recruitment marker, *Ccl2*, a strong chemo-attractant of macrophages, and *Tnf*, a pro-inflammatory cytokine, were strongly correlated with body weight and fat mass only in EF of obese mice on the HFD. The role of macrophages in AT still remains unclear; it is speculated that they might participate in tissue remodeling for angiogenesis [10,24]. Expression levels of angiogenic genes, except *Pecam1*, were lower or indifferent in IF of HFD-fed mice relative to LFD-fed mice, while macrophage-related genes were expressed at higher levels, although their overall expression level was lower relative to EF. This supports a differential ability of tissue remodeling between the depots. Given the overall lower expression levels of genes related to hypoxia, angiogenesis, and pro-inflammation along with lower angiogenic capacity in IF, there is likely to be a lower demand or ability for tissue remodeling in subcutaneous fat compared to visceral fat (Figure 5).

5. CONCLUSIONS

The AT depot is differentially correlated with insulin resistance and other diseases, including cardiovascular disease [17]. Visceral fat has greater inflammatory status than subcutaneous fat and is more associated with metabolic diseases [17]. Recently, the critical role of AT angiogenesis has been raised in obesity, diabetes, and other fields, including therapeutic approaches for neo-vascularization or implantation with adipose-derived stem cells [1,3,15,16,28]. The present study indicated that visceral fat had higher angiogenic capability in response to HFD-induced over-nutrition than subcutaneous fat. Therefore, further studies based on these findings would enable us to better understand the depot-specific function of AT and its therapeutic applications related to angiogenesis.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.09.001.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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