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ORIGINAL ARTICLE

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FSTL3 is highly expressed in adipose tissue of individuals with overweight or obesity and is associated with inflammation

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Abstract

Objective: This study aimed to investigate the expression of follistatin-like 3 (FSTL3) in adipose tissue in individuals with overweight or obesity and to explore the role of FSTL3 in human adipocytes, as well as the relationship between serum FSTL3 levels and fat distribution and inflammation.

Methods: This study enrolled 236 individuals (171 with overweight or obesity; aged 18–67 years). Bulk transcriptome sequencing was performed on subcutaneous and visceral adipose tissue. The function of FSTL3 was studied in human adipocytes. Serum FSTL3 levels were measured using enzyme-linked immunosorbent assay.

Results: Adipose FTSL3 expression was higher in individuals with overweight or obesity than in individuals with normal weight. FSTL3 was mainly expressed in mature adipocytes and stimulated by tumor necrosis factor alpha (TNFα). FSTL3 suppressed inflammatory responses in human adipocytes, whereas FSTL3 knockdown promoted inflammatory responses. Serum FSTL3 levels were correlated with adipose FTSL3 expression and obesity-related indicators (all $p < 0.05$). Multiple linear regression analysis showed that serum FSTL3 levels were independently associated with the visceral fat area and serum TNFα levels (both $p < 0.05$).

Conclusions: FSTL3 was highly expressed in adipose tissue in individuals with overweight or obesity and could suppress adipocyte inflammation. Serum FSTL3 levels might be considered as a biomarker of visceral obesity and inflammation.

INTRODUCTION

Obesity, particularly visceral obesity, carries a high risk of developing metabolic disorders [1]. Adipose tissue is accompanied by chronic low-grade inflammation with the increased secretion of proinflammatory cytokines [2, 3], which contribute to obesity-associated metabolic complications [2].

Follistatin-like 3 (FSTL3) is a secreted glycoprotein belonging to the follistatin protein family and it is primarily expressed in the placenta as well as in the testis, heart, adipose tissue, and pancreas. It is an extracellular inhibitor of the transforming growth factor beta (TGF-β) family ligands, including activin, myostatin, and bone morphogenetic proteins [2]. Several previous investigations have explored the role of FSTL3 in human metabolic diseases. A study based on European individuals showed that circulating FSTL3 levels were higher in individuals with obesity and positively associated with body mass index (BMI), fat

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mass (FM), and waist circumference (WC) [4]. Circulating FSTL3 levels decreased in response to energy deprivation [5] and physical activity [6]. Furthermore, the expression of FSTL3 decreased in human subcutaneous adipose tissue (SAT) after dietary restrictions [7]. These studies suggested that metabolic status regulated FSTL3. It was revealed that muscle-specific overexpression of FSTL3 reduced fat accumulation and improved insulin sensitivity during high-fat feeding [8], whereas other studies reported that FSTL3 knockout mice exhibited decreased epididymal white adipose tissue mass through activin and myostatin bioactivity [9, 10], which linked FSTL3 to fat deposition. Although the mechanism underlying the role of FSTL3 on adipose tissue remains unidentified, the relationship between FSTL3 and metabolic diseases has garnered increasing interest.

To further investigate the relationship between FSTL3 and metabolic diseases, abdominal fat distribution was also included in this study. Fat distribution (including subcutaneous and visceral fat), especially visceral adipose tissue (VAT), greatly determines metabolic morbidity and mortality [11, 12]. Several indicators of adiposity are frequently used to define overweight and obesity, such as BMI, WC, and FM, and although convenient and noninvasive, they cannot reflect the accurate abdominal fat distribution. SAT and VAT can be accurately measured using magnetic resonance imaging (MRI), with subcutaneous fat area (SFA) and visceral fat area (VFA) as calculated indices. Therefore, elucidating the relationships between FSTL3 and SFA and VFA is important for investigating metabolic diseases and developing targeted therapeutic approaches.

Thus, our study was designed to investigate whether the expression of FSTL3 is dysregulated in individuals with overweight or obesity, the potential function of FSTL3 in adipocytes, and the relationship between serum FSTL3 levels and VFA.

Study Importance

What is already known?

- Circulating follistatin-like 3 (FSTL3) levels are associated with altered metabolic status, including inflammation, obesity, exercise, and metabolic surgery. In addition, FSTL3 expression in human subcutaneous adipose tissue (SAT) decreased after dietary restriction.
- FSTL3 knockout mice showed decreased epididymal adipose tissue mass and improved glucose tolerance. FSTL3 overexpression reduced fat accumulation and improved insulin sensitivity of high-fat diet-fed mice.

What does this study add?

- FSTL3 expression was upregulated in human visceral adipose tissue (VAT) in individuals with overweight or obesity, in addition to increased expression in SAT.
- FSTL3 was mainly expressed in mature adipocytes and regulated by inflammation, and it played a role in inhibiting inflammation.
- Serum FSTL3 was independently associated with VAT accumulation and inflammatory factor TNFα.

How might these results change the direction of research or the focus of clinical practice?

- This is the first human evidence for higher FSTL3 expression in VAT in individuals with overweight or obesity.
- The finding that FSTL3 relieved adipocyte inflammation encourages further research into underlying mechanisms and treatment targets.
- Serum FSTL3 accurately identified the obesity state with inflammation and fat distribution, which could identify patients at increased risk for metabolic disorder progression.

METHODS

Study population

This study enrolled 236 participants (aged 18–67) who underwent either elective abdominal surgery for cholecystectomy or weight reduction bariatric surgery at the Shanghai Jiao Tong University School of Medicine Affiliated Sixth People's Hospital in Shanghai, China, between July 2019 and August 2020. All participants provided written informed consent for participation and received standardized questionnaires (including history of illness, medication, and lifestyle habits), physical examinations, and laboratory tests. The exclusion criteria were as follows: use of insulin or lipid-lowering drugs; presence of malignant tumor, acute infection, moderate to severe anemia, severe hepatic and renal dysfunction, heart failure, respiratory failure, gastrointestinal ulcers, or inflammatory bowel disease; or hormone replacement therapy. SAT and VAT samples were obtained from all participants. This study was approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine Affiliated Sixth People's Hospital.

The participants were diagnosed as overweight or obesity based on the criterion (BMI ≥ 25 kg/m^2) prescribed by the 1999 World Health Organization guidelines [13]. Diabetes mellitus was diagnosed based on the 2021 criteria of the American Diabetes Association [14]. Hypertension was defined according to the 2020 guidelines of the International Society of Hypertension [15]. Dyslipidemia was diagnosed according to the 2016 China Adult Dyslipidemia Prevention Guidelines [16].

Anthropometric and biochemical measurements

Among 236 individuals, detailed clinical information and serum were available for 198. Height was measured with a stadiometer and

weight with a calibrated electronic scale with standard methods according to our previous study [17]. BMI was calculated as weight in kilograms divided by height in meters squared. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using an electronic sphygmomanometer (Omron). WC was measured to the nearest centimeter at the midway level between the iliac crest and the lowest rib with the participants standing. Venous blood samples were drawn from all participants after a 10-hour overnight fast in the morning. The following biochemical indices were measured: fasting plasma glucose (FPG), 2-hour postprandial plasma glucose, glycosylated hemoglobin (HbA_{1c}), fasting insulin (FINS), blood lipids (total cholesterol [TC], triglycerides [TG], high-density lipoprotein cholesterol [HDL-c], and low-density lipoprotein cholesterol [LDL-c]), serum free fatty acids (FFAs), C-reactive protein (CRP), tumor necrosis factor alpha (TNFα), and interleukin 6 (IL-6). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows: HOMA-IR = FINS (mU/L) × FPG (mmol/L)/22.5. Adipose tissue insulin resistance (Adipo-IR) was the product of FFAs (mmol/L) × FINS (pmol/L).

Serum FSTL3 levels were quantified using enzyme-linked immunosorbent assay (ELISA) kits (DFLRG0, R&D Systems) [4, 18], with intra- and interassay variations of 3.95% and 7.05%, respectively. ELISA was also performed to detect serum leptin levels (DLP00, R&D Systems), with intra- and interassay variations of 3.27% and 6.59%, respectively.

Measurement of FM and abdominal fat distribution

Total FM and body fat percentage (fat%) were estimated using a fully automatic bioelectrical impedance body composition analyzer (TBF-418B, Tanita Corp). SFA and VFA were measured using an MRI scanner (Archiva 3.0 T, Philips Medical Systems). The specific methods used have been described in our previous study [19].

RNA sequencing of human abdominal adipose tissue

SAT and VAT were harvested from all the 236 participants and snap frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). The integrity of total RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc.). Samples with RNA integrity number values above 7.0 were used for RNA sequencing (RNA-seq). RNA-seq strand-specific libraries were constructed using a VAHTS Total RNA-seq (H) Library Prep Kit according to the manufacturer's instructions. The samples were sequenced using the NovaSeq 6000 platform (Illumina). Raw sequencing data were transformed into fastq format. After quality filtering, the sequencing data were used to analyze coding gene expression. Gene abundance was expressed as fragments per kilobase of exon per million reads mapped (FPKM). Library construction and sequencing were performed by Sinotech Genomics Co. Ltd.

Peripheral blood mononuclear cell isolation from human whole blood

For human peripheral blood mononuclear cell (PBMC) isolation, whole blood samples were drawn from seven individuals with normal weight and seven individuals with overweight or obesity. PBMCs were isolated by a standard method (Hypaque–Ficoll, GE Healthcare). Detailed methods are provided in the online Supporting Information.

Primary human adipose stromal cell culture and treatment

Human primary adipocytes were extracted from SAT samples and differentiated according to the standard procedures. Detailed methods are provided in the online Supporting Information. The cell culture supernatants were collected after cells were fully differentiated and treated. The FSTL3 levels were quantified using the ELISA previously mentioned. C-C motif chemokine 2 (CCL2) and IL-6 levels were quantified using ELISA kits (VAL134 R&D and VAL102, R&D Systems).

Isolation and culture of human adipose tissue blocks

For *ex vivo* adipose tissue block treatment, SAT and VAT samples were cut into 20-mg fat pads and stimulated in medium with or without 50-ng/mL TNFα for 24 hours. The medium was collected to determine FSTL3 levels.

Transient transfection

On day 12 of human primary adipocyte differentiation, cells were transfected with small interfering RNA (siRNA) targeting FSTL3 and the control siRNA using Lipofectamine RNAiMAX Transfection Reagent (13778150; Thermo Fisher, USA) according to the manufacturer's instruction. Cells were harvested for RNA extraction after 48 hours. The cell culture supernatants for ELISA and protein samples were collected after 72 hours. siRNA used in this study was synthesized and purchased from GenePharma (Supporting Information Table S1).

RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was isolated using Trizol Reagent (15596018; Invitrogen) according to the manufacturer's instructions. RNA was converted to complementary DNA (cDNA) using the GoScript Reverse Transcriptase Kit (A5001, Promega). Real-time quantitative polymerase chain reaction amplification (qPCR) was conducted using TB Green Premix Ex Taq II (RR820A, Takara) on a LightCycler 480 System (Bio-Rad). The primer sequences used are summarized in Supporting Information Table S1.

Western blot

The primary antibodies for detecting FSTL3 (0.1 μg/mL; AF1288-SP, R&D Systems) and tubulin (dilution: 1:1000; 2144S, Cell Signaling Technology) were diluted using antibody dilution buffer (C520011, Sangon Biotech) and were incubated overnight at 4 °C. Then the membrane was incubated with the second antibody (dilution: 1:5000; anti-rabbit/goat IgG, horseradish peroxidase conjugate, Cell Signaling Technology) for 1 hour at room temperature. The membrane was washed three times with Tris-buffered saline with Tween 20 after each incubation and visualization was carried out using the Bio-Rad chemiluminescent imaging system.

Statistical analyses

All statistical analyses were performed using SPSS Statistics version 25.0 (IBM Corp.). The normal distribution of data was analyzed using the one-sample Kolmogorov–Smirnov test. After the normality test, the normally distributed data were expressed as mean (standard deviation), whereas the skewed variables were presented as median (interquartile range, 25%–75%). Categorical variables were described using the constituent ratio. Group comparisons were performed by conducting a nonpaired *t* test or paired *t* test for the normally distributed data and Wilcoxon rank sum test for the skewed data. Correlations between data were performed using Spearman correlation. Multivariate linear regression analysis was used to explore the factors influencing serum FSTL3 levels. The level of statistical significance was set at a two-tailed p value < 0.05.

RESULTS

Increased expression of FSTL3 in adipose tissue in individuals with overweight or obesity

To examine the tissue expression profile of FSTL3, we first analyzed a published available human tissue proteomics database [20] and found that FSTL3 was highly expressed in adipose tissue (Supporting Information Figure S1). Higher FSTL3 expression in adipose tissue has also been confirmed by a previous study [21]. Then we performed high-throughput transcriptome sequencing of paired SAT and VAT of 236 participants (including 65 normal-weight individuals and 171 individuals with overweight or obesity) to further identify the FSTL3 expression profile. Among the total individuals, 71 (30.08%) had diabetes (Supporting Information Table S2). A slightly higher expression was observed in SAT compared with VAT (Supporting Information Figure S1).

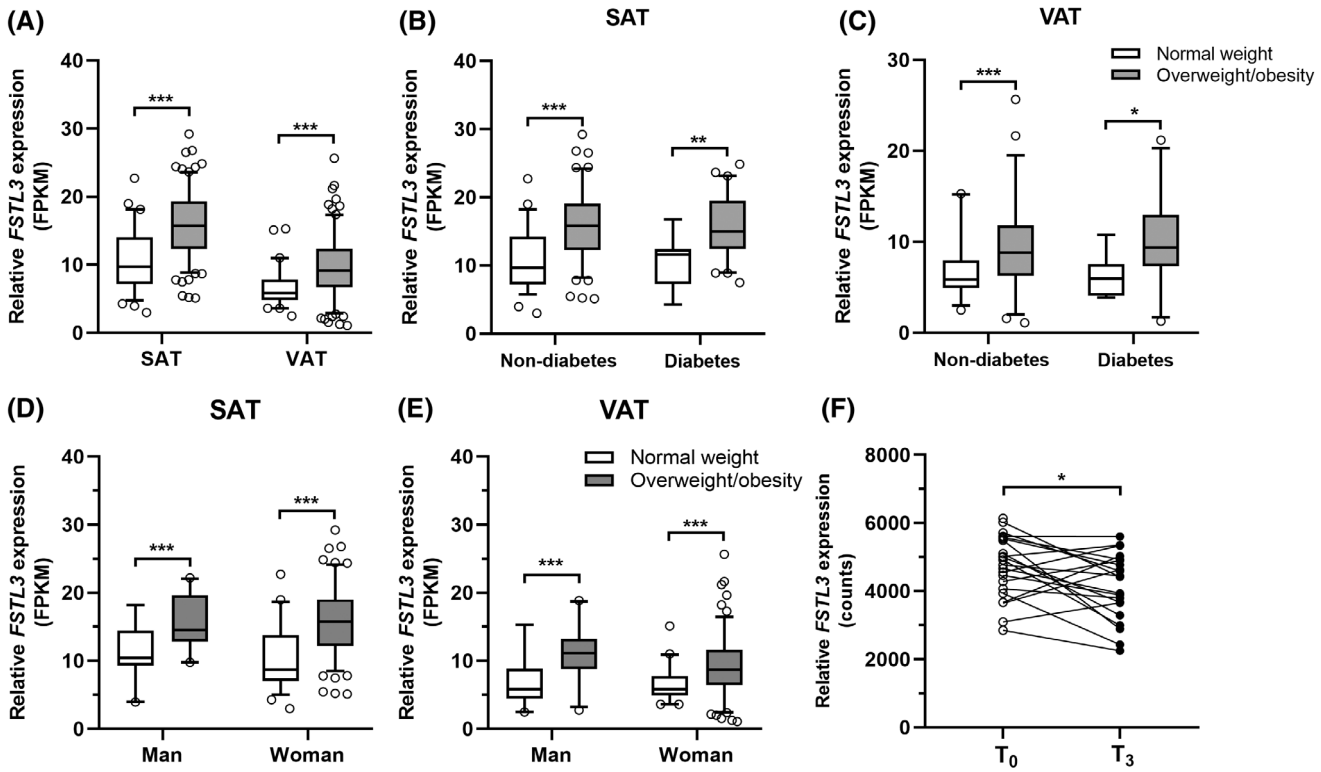

FIGURE 1 Adipose FSTL3 mRNA expression upregulated in individuals with overweight or obesity. (A) Comparison of FSTL3 expression levels in SAT and VAT between normal-weight individuals and individuals with overweight or obesity. (B, C) Comparison of FSTL3 expression levels in adipose tissue of individuals with and without diabetes and normal weight and overweight or obesity. (D, E) Comparison of FSTL3 expression levels in adipose tissue of men and women with normal weight and overweight or obesity. (F) Changes of FSTL3 expression levels in human SAT 3 months after bariatric surgery. Data are shown as median with 25th and 75th percentiles. *p < 0.05, **p < 0.01, ***p < 0.005. FPKM, fragments per kilobase of exon per million reads mapped; FSTL3, follistatin-like 3; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue

Compared with normal-weight individuals, adipose tissue FSTL3 expression was significantly higher in individuals with overweight or obesity (both $p < 0.001$, Figure 1A). Additionally, the results were consistent for the subgroups created based on diabetes and gender (both $p < 0.05$, Figure 1B–E). Consistently, there was a downregulation of FSTL3 expression in human SAT at 3 months after Roux-en-Y gastric bypass derived from the data set from Poitou et al. [22] (Figure 1F).

Expression of FSTL3 in different cell fractions of human abdominal adipose tissue

To further determine the expression of FSTL3, we isolated the stromal vascular fraction (SVF) and mature fraction (MF) from SAT and VAT and observed an absolutely higher mRNA expression of FSTL3 in MF (Figure 2A). Because the differentiation capacity of subcutaneous SVF was reported to be higher compared with visceral SVF [23], we next extracted and differentiated SVF from SAT. Increased expression of adipocyte differentiation markers (peroxisome proliferator activated receptor gamma [PPARγ] and fatty acid binding protein 4 [FABP4]) were accompanied by increased FSTL3 mRNA expression (Figure 2B–D). PBMCs are a critical part of the immune system in the obesity state [24]. We then isolated PBMCs from 14 individuals (aged 18–45). The clinical characteristics are shown in Supporting Information Table S3. FSTL3 expression was barely detectable in PBMCs (Supporting Information Table S4), which indicated that the nonimmune composition of adipose tissue participated in the expression of FSTL3. The aforementioned results suggested that FSTL3 was mainly expressed in human mature adipocytes.

Gene coexpression analysis of adipose FSTL3

The aforementioned finding prompted us to explore the potential regulatory factors and function of FSTL3 in human primary adipocytes. With this aim, genome-wide coexpression analysis was performed to predict pathways associated with altered FSTL3 expression in human SAT and VAT. Through analyzing whole-transcriptome sequencing data, we found that genes positively coexpressed with FSTL3 showed a significant enrichment in inflammation pathways, whereas gene expression in VAT was more enriched than in SAT (Figure 3A). Among these pathways, TNF signaling, toll-like receptor signaling, and nuclear

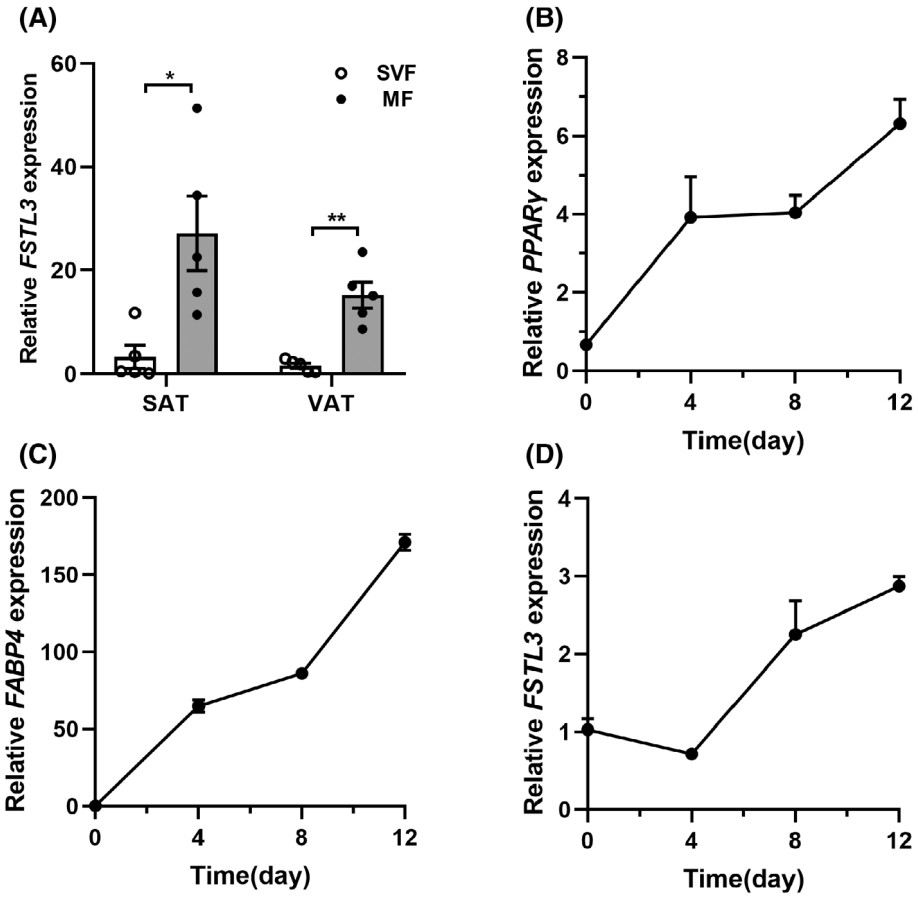

FIGURE 2 FSTL3 expressed mainly in mature adipocytes. (A) FSTL3 mRNA expression in SVF and MF extracted components of human SAT and VAT. Data are presented as mean ± SEM for independent experiments ($n = 4–5$ individuals). (B–D) Expression of adipocyte differentiation markers (PPARγ and FABP4) and FSTL3 during differentiation of human primary adipose stromal cells. Data are shown as median with 25th and 75th percentiles. *$p < 0.05$, ** $p < 0.01$. FABP4, fatty acid binding protein 4; FSTL3, follistatin-like 3; MF, mature fraction; PPARγ, peroxisome proliferator activated receptor gamma; SAT, subcutaneous adipose tissue; SVF, stromal vascular fraction; VAT, visceral adipose tissue

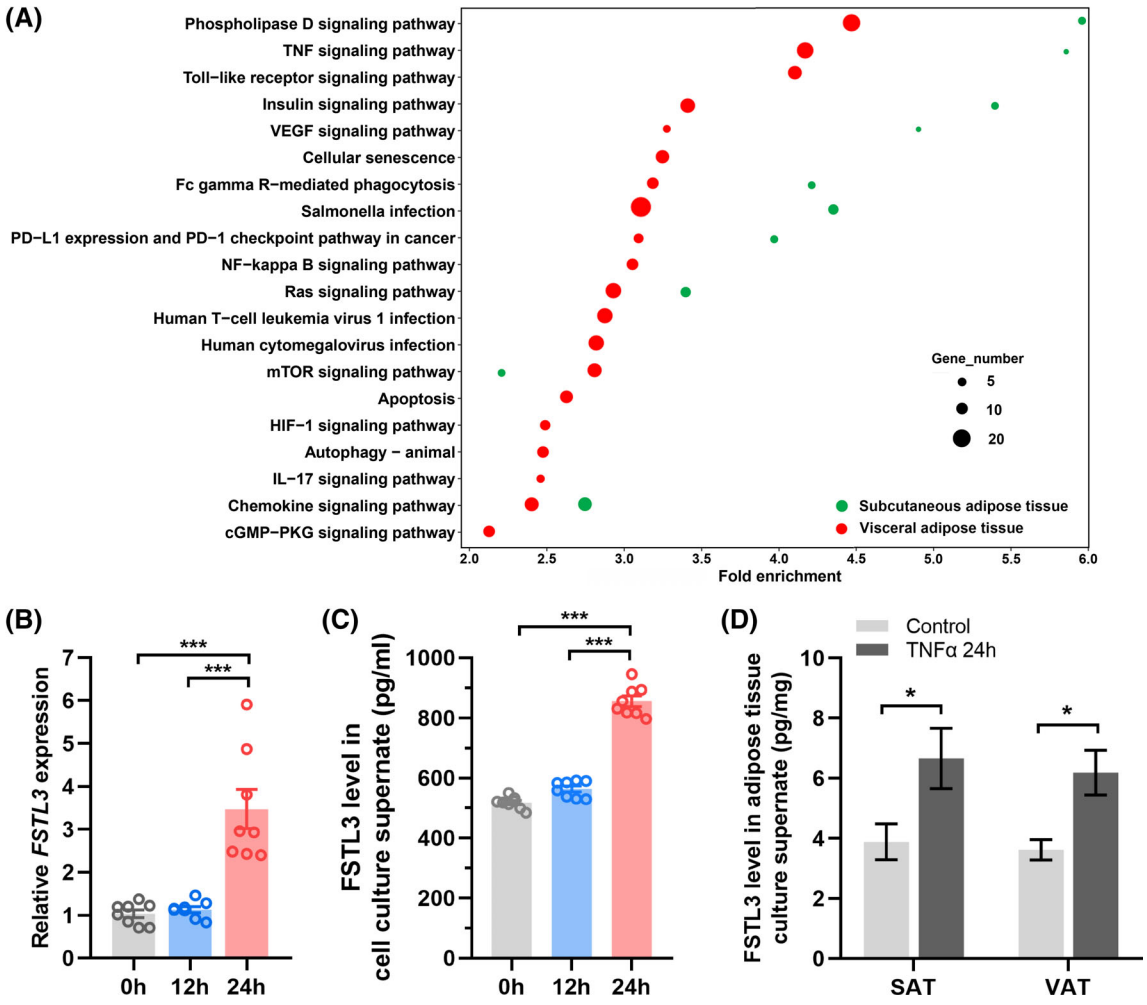

FIGURE 3 High FSTL3 expression associated with proinflammatory pathways in human adipose tissue. (A) FSTL3 mRNA levels in SAT or VAT were correlated with other transcript levels globally (Illumina transcription sequencing analysis, log2-transformed signal intensities). Genes positively coexpressed with FSTL3 (Spearman $r \geq 0.4$) were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. (B) FSTL3 mRNA expression in differentiated human primary adipose stromal cells treated with TNFα (50 ng/mL) for 12 and 24 hours. (C) FSTL3 levels in the cell culture supernatant of mature adipocytes treated with TNFα (50 ng/mL) for 12 and 24 hours. (D) FSTL3 levels in the supernatant of SAT and VAT treated with TNFα (50 ng/mL) for 24 hours. $^{*}p < 0.05$, $^{***}p < 0.005$. FSTL3, follistatin-like 3; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue [Color figure can be viewed at wileyonlinelibrary.com]

factor-κB (NF-κB) signaling pathways have been implicated in chronic inflammation in adipose tissue [25]. The coexpression analyses also indicated a link to hypoxia-inducible factor-1 (HIF-1) signaling, which mediated the effects of adipocyte oxygen consumption on inflammation and insulin resistance [26]. The expression of inflammation-related genes showed consistent changes with FSTL3 expression (Supporting Information Figure S2), indicating the likelihood that FSTL3 was involved in the inflammatory process. In addition, we analyzed the association between FSTL3 expression and proinflammatory cytokine, adipogenic, lipogenic, lipolytic, lipid droplet development, and mitochondrial biogenesis–related genes (Supporting Information Table S5). In addition to a significant correlation with proinflammatory genes, lipolytic and mitochondrial biogenesis–related genes also exhibited some degree of correlation with FSTL3 expression, and we focused on the role of FSTL3 in adipocyte inflammation in this study.

To identify the possibility that a proinflammatory milieu could contribute to regulating FSTL3 expression, we treated differentiated human primary adipocytes with proinflammatory factor TNFα (50 ng/mL) for 12 or 24 hours. Then we measured increased mRNA expression and secretion levels of FSTL3 (Figure 3B,C). Simultaneously, elevated FSTL3 levels were detected in the tissue culture supernatant from isolated SAT and VAT treated with TNFα (50 ng/mL) for 24 hours (Figure 3D). Our findings suggested that inflammatory factor enhanced the expression and secretion of FSTL3.

Effects of recombinant FSTL3 on adipogenesis and inflammatory gene expression in human primary adipocytes

To further explore the role of FSTL3, we differentiated human primary adipocytes and assessed whether recombinant FSTL3 protein could affect cell differentiation and inflammation status. After

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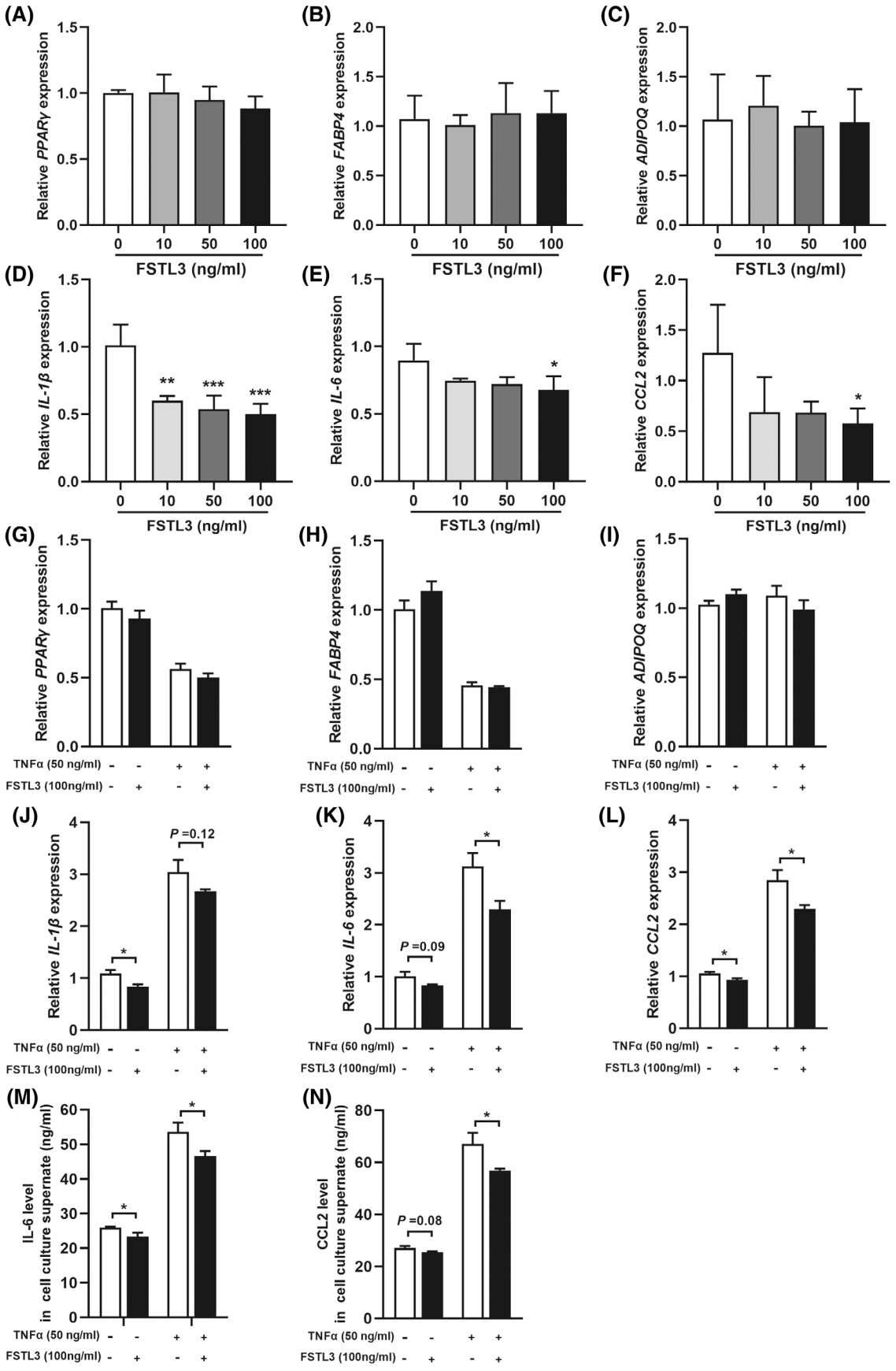

FIGURE 4 Legend on next page.

treating adipocytes with recombinant FSTL3 of different concentrations (0, 10, 50, 100 ng/mL) for 24 hours, we detected the expression of adipogenesis genes (PPARγ, FABP4, and adiponectin [ADIPOQ]) was not altered (Figure 4A–C). Of note, recombinant FSTL3 downregulated the expression of IL-1β, IL-6, and CCL2 (Figure 4D–F). These results indicated that FSTL3 might inhibit the adipocyte inflammatory response to a certain extent through autocrine action.

We also performed experiments to evaluate the combined effect of TNFα and recombinant FSTL3 treatment on adipocyte inflammation. Human primary adipocytes were pretreated with recombinant FSTL3 (100 ng/mL) for 12 hours, followed by 24-hour treatment with TNFα (50 ng/mL) in the presence of FSTL3. We found that FSTL3 had no effect on adipogenic genes with or without TNFα stimulation (Figure 4G–I). Moreover, recombinant FSTL3 inhibited the expression of IL-6 and CCL2 and showed a tendency for IL-1β inhibition upon TNFα stimulation (Figure 4J–L). Because of the abundant expression and secretion of IL-6 and CCL2 in adipocytes [3], we also detected increased IL-6 and CCL2 levels by the treatment of TNFα alone and decreased IL-6 and CCL2 levels cotreated with recombinant FSTL3 (Figure 4M,N). These results further strengthen the anti-inflammatory role of FSTL3.

Effects of FSTL3 knockdown on adipocyte inflammation

We performed the FSTL3 knockdown assay in differentiated human primary adipocytes using siRNA. The knockdown efficiency was verified by qPCR and Western blot experiments (Figure 5A,B). Additionally, the secreted FSTL3 levels in culture supernatant were decreased (Figure 5C). The cellular morphology and expression of adipogenic genes (PPARγ, FABP4, and ADIPOQ) showed no difference between the control and FSTL3 knockdown group (Figure 5D, E). The aforementioned results suggested a successful knockdown of FSTL3, and siRNA-mediated transfection did not affect adipocyte differentiation. Then knockdown of FSTL3 increased the expression of inflammatory genes, including IL-1β, IL-6, and CCL2, with or without TNFα stimulation (Figure 5F–H). Elevated secretion of IL-6 and CCL2 was also detected (Figure 5I,J). Therefore, FSTL3 knockdown exhibited an inflammatory status, which demonstrated that FSTL3 played a critical regulatory role in adipocyte inflammation.

Association between serum FSTL3 levels and obesity-related indicators

Next, we attempted to determine the relationship between serum FSTL3 levels and FSTL3 expression in adipose tissue. Serum samples and detailed clinical information were available from 198 (43 men and 155 women, aged 18–61) of 236 individuals. The clinical characteristics are shown in Table 1. Among 198 participants, 160 (80.8%) were overweight or had obesity, 63 (31.8%) had diabetes, 37 (18.7%) had hypertension, and 20 (10.1%) had dyslipidemia. Furthermore, serum FSTL3 levels were consistent with FSTL3 expression in both SAT ($r = 0.202$, $p = 0.004$) and VAT ($r = 0.187$, $p = 0.009$) (Figure 6A,B). Because smoking was well known to affect inflammatory status [27], we further adjusted for smoking status and found that the correlation still existed in SAT and VAT (Supporting Information Table S6). The median levels of serum FSTL3 were higher in individuals with overweight or obesity compared with individuals with normal weight (3413.94 [1850.84–5555.89] pg/mL vs. 4104.96 [3137.20–5129.47] pg/mL, $p = 0.036$), whereas no difference was observed between the nondiabetic and diabetic populations (3858.56 [2953.91–5062.01] pg/mL vs. 4306.47 [3151.34–5473.79] pg/mL, $p = 0.069$).

Correlation analyses revealed that serum FSTL3 levels were positively associated with BMI, WC, FM, fat%, FPG, HbA_{1c}, HOMA-IR, adipo-IR, serum leptin levels, and inflammation mediators (CRP, IL-6 and TNFα; all $p < 0.05$, Figure 6C). The associations remained after adjusting for smoking habits (Supporting Information Table S6). Among individuals with overweight and obesity, multivariate stepwise regression analysis was conducted, which defines serum FSTL3 levels as a dependent variable and age, gender, smoking status, BMI, WC, FM, HOMA-IR, CRP, IL-6, TNFα, SFA, and VFA as the independent variables. The results indicated that VFA (standardized $\beta = 0.364$, $p < 0.001$) and TNFα (standardized $\beta = 0.65$, $p = 0.049$) were independently associated with serum FSTL3 levels (Table 2).

DISCUSSION

To the best of our knowledge, this is the first study to report the higher expression of FSTL3 in SAT and VAT among individuals with overweight or obesity, which is based on sequencing data with a large sample size. We uncovered that FSTL3 was secreted by human mature adipocytes and functioned during inflammatory responses. Additionally, this study identified the correlation between serum

FIGURE 4 Effect of recombinant FSTL3 on adipocyte inflammatory response. (A–C) Expression of adipocyte differentiation markers (PPARγ, FABP4, and ADIPOQ) in human primary adipocytes after treatment with recombinant FSTL3 (10, 50, and 100 ng/mL) for 24 hours. (D–F) Expression of adipocyte inflammatory genes (IL-1β, IL-6, and CCL2) in human primary adipocytes after treatment with recombinant FSTL3 (10, 50, and 100 ng/mL) for 24 hours. (G–I) Expression of adipocyte differentiation markers (PPARγ, FABP4, and ADIPOQ) in human primary adipocytes with or without FSTL3 (100 ng/mL) treatment and TNFα (50 ng/mL) stimulation. (J–L) Expression of adipocyte inflammatory genes (IL-1β, IL-6, and CCL2) in human primary adipocytes with or without FSTL3 (100 ng/mL) treatment and TNFα (50 ng/mL) stimulation. (M, N) IL-6 and CCL2 levels in the cell culture supernatant of mature adipocytes with or without FSTL3 (100 ng/mL) treatment and TNFα (50 ng/mL) stimulation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. ADIPOQ, adiponectin; CCL2, C-C motif chemokine 2; FABP4, fatty acid binding protein 4; FSTL3, follistatin-like 3; IL, interleukin; PPARγ, peroxisome proliferator activated receptor gamma

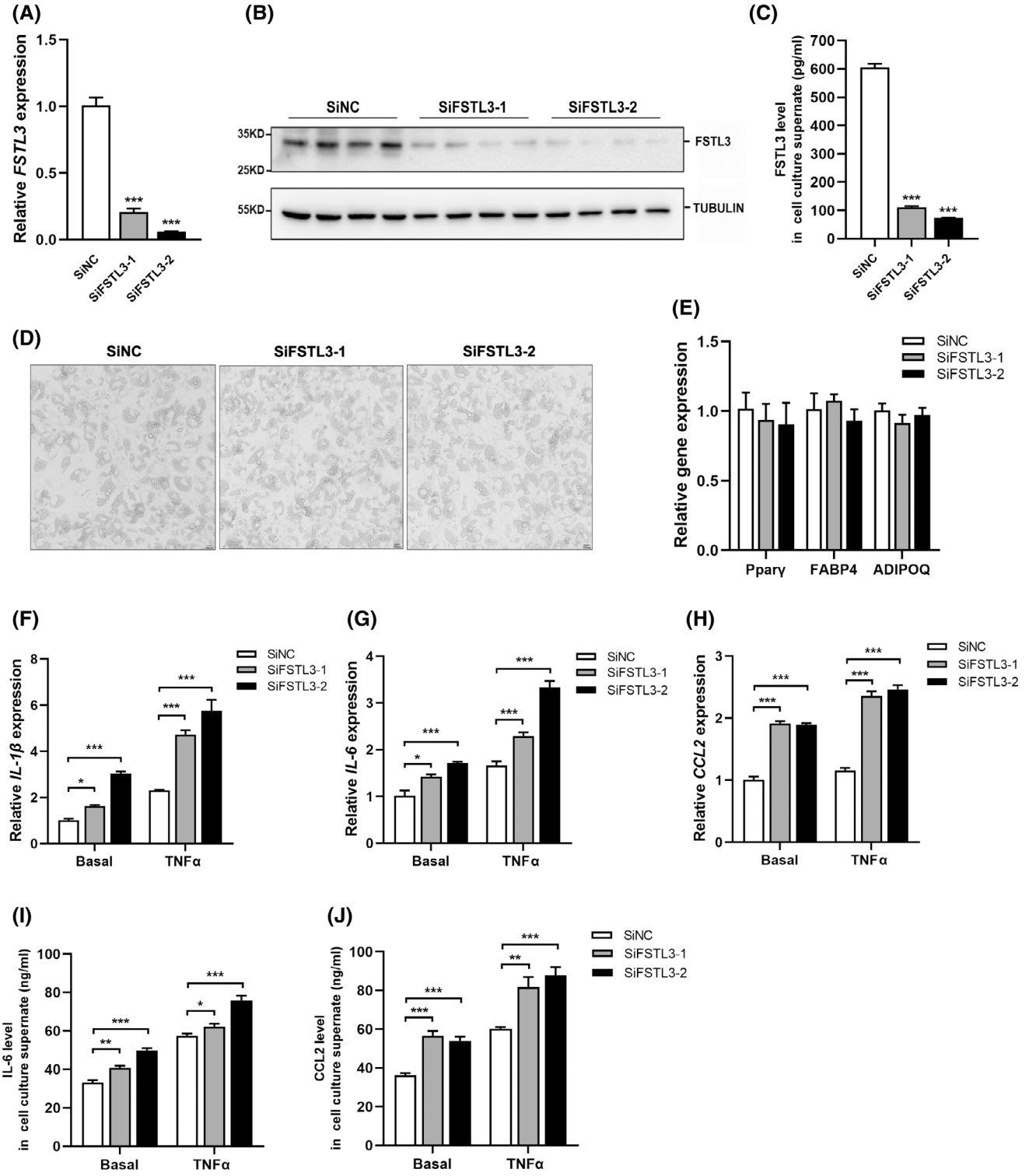

FIGURE 5 Effects of FSTL3 knockdown on adipocyte inflammation. (A) FSTL3 mRNA expression of human primary adipocytes after FSTL3 knockdown. (B) Western blots of FSTL3 in human primary adipocytes after FSTL3 knockdown. (C) FSTL3 levels in the cell culture supernatant of mature adipocytes after FSTL3 knockdown. (D) Bright-field images of FSTL3 knockdown and control human primary adipocytes. Representative of $n = 8$. (E) Expression of adipocyte differentiation markers (PPARγ, FABP4, and ADIPOQ) in FSTL3 knockdown and control human primary adipocytes. (F–H) Expression of adipocyte inflammatory genes (IL-1β, IL-6, and CCL2) in FSTL3 knockdown and control human primary adipocytes. (I–J) IL-6 and CCL2 levels in the cell culture supernatant of FSTL3 knockdown and control human primary adipocytes. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. ADIPOQ, adiponectin; CCL2, C-C motif chemokine 2; FABP4, fatty acid binding protein 4; FSTL3, follistatin-like 3; IL, interleukin; PPARγ, peroxisome proliferator activated receptor gamma

TABLE 1 Clinical characteristics of participants

Variable	Normal weight ($n = 38$)	Overweight/obesity ($n = 160$)	p value
Men/women	9/29	34/126	–
Age (y)[a]	42.0 ± 9.9	38.3 ± 7.3	0.390
BMI (kg/m^2)	22.03 (20.92–22.99)	38.40 (32.70–42.25)	<0.001
WC (cm)[a]	81.04 ± 6.78	118.45 ± 16.53	<0.001
FM (kg)	16.20 (12.85–18.03)	50.70 (38.30–62.78)	<0.001
Fat %[a]	26.10 ± 5.31	48.14 ± 9.47	0.001
SBP (mmHg)	120 (113–128)	130 (120–140)	<0.001
DBP (mmHg)[a]	76 ± 11	84 ± 12	0.301
TC (mmol/L)[a]	4.66 ± 0.77	4.98 ± 1.09	0.093
TG (mmol/L)	1.29 (0.67–1.56)	1.71 (1.25–2.29)	<0.001
HDL-c (mmol/L)	1.22 (0.97–1.59)	1.07 (0.88–1.34)	0.046
LDL-c (mmol/L)[a]	2.98 ± 0.91	3.04 ± 0.93	0.615
FFAs (μEq/L)[a]	535.37 ± 223.66	603.22 ± 216.37	0.591
FPG (mmol/L)	4.97 (4.55–5.33)	5.66 (5.14–6.72)	<0.001
HbA_{1c} (%)	5.40 (5.30–5.63)	5.90 (5.50–6.78)	<0.001
HOMA-IR	1.36 (0.93–2.07)	7.74 (4.53–12.26)	<0.001
CRP (mg/L)	0.37 (0.16–1.06)	4.47 (1.83–7.81)	<0.001
IL-6 (pg/mL)	1.69 (0.75–3.62)	3.94 (2.07–6.44)	<0.001
TNFα (pg/mL)	5.55 (4.33–6.45)	7.00 (5.60–8.98)	<0.001
Leptin (ng/mL)	5.17 (2.36–14.40)	46.85 (27.78–73.67)	<0.001
ADIPO-IR (mmol/L × pmol/L)	17.64 (9.17–41.42)	99.56 (54.69–161.92)	<0.001
Serum FSTL3 (pg/mL)	3413.94 (1850.84–5555.89)	4104.96 (3137.20–5129.47)	0.036
Noninsulin glucose-lowering therapies, n (%)	0 (0)	22 (13.75)	<0.001
Antihypertensive therapies, n (%)	1 (2.63)	26 (16.25)	<0.001
Lipid-regulating drug, n (%)	0 (0)	3 (1.88)	<0.001
Current smoking, n (%)	1 (2.63)	15 (9.38)	<0.001

Abbreviations: ADIPO-IR, adipose tissue insulin resistance; CRP, C-reactive protein; DBP, diastolic blood pressure; FFAs, free fatty acids; FM, fat mass; FPG, fasting plasma glucose; FSTL3, follistatin-like 3; HDL-c, how-density lipoprotein cholesterol; HbA_{1c}, glycosylated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance; IL-6, interleukin 6; LDL-c, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; TNFα, tumor necrosis factor α; WC, waist circumference.

[a]Normally distributed variables are expressed as mean (standard deviation). Other skewed distributed variables are expressed as median (interquartile range).

FSTL3 levels and adipose FSTL3 expression. We also found that visceral adiposity and inflammatory factor TNFα were independently related to serum FSTL3 levels in Chinese individuals with overweight or obesity.

FSTL3 was first reported as a TGF-β family binding protein in 2000 and it is mainly bound to TGF-β family members (including activin and myostatin) to inhibit their transcriptional activity [28]. However, little is known about the expression profile of FSTL3 in human SAT and VAT. Only one study based on human SAT samples reported that FSTL3 expression decreased after dietary restriction in individuals with severe obesity [7]. We identified the higher FSTL3 expression in SAT and VAT in individuals with overweight or obesity, with the largest subcutaneous and visceral sample sizes applied to this question. To further identify the expression of FSTL3 in cell fractions of adipose tissue, we isolated SVF and MF from SAT and VAT, extracted PBMCs, and differentiated human primary adipocytes. Our results showed that FSTL3 was highly expressed in MF. In addition, FSTL3 expression levels increased upon human adipocyte differentiation.

Accumulating evidence reveals that inflammatory responses of adipose tissue is a central and reversible mechanism through which obesity promotes the risk of metabolic complications [29]. Impaired secretion of adipokines owing to adipose tissue dysfunction led to the pathogenesis of obesity-linked complications. Previous studies showed that FSTL3 was involved in muscle function and hepatic glycolipid metabolism [6, 8]. FSTL3-overexpressing mice had decreased fat accumulation and improved insulin sensitivity [8], whereas homozygous FSTL3 knockout mice showed decreased VAT and improved glucose tolerance [9, 10]. The regulation of FM might be mediated by myostatin because previous research showed that loss or gain of

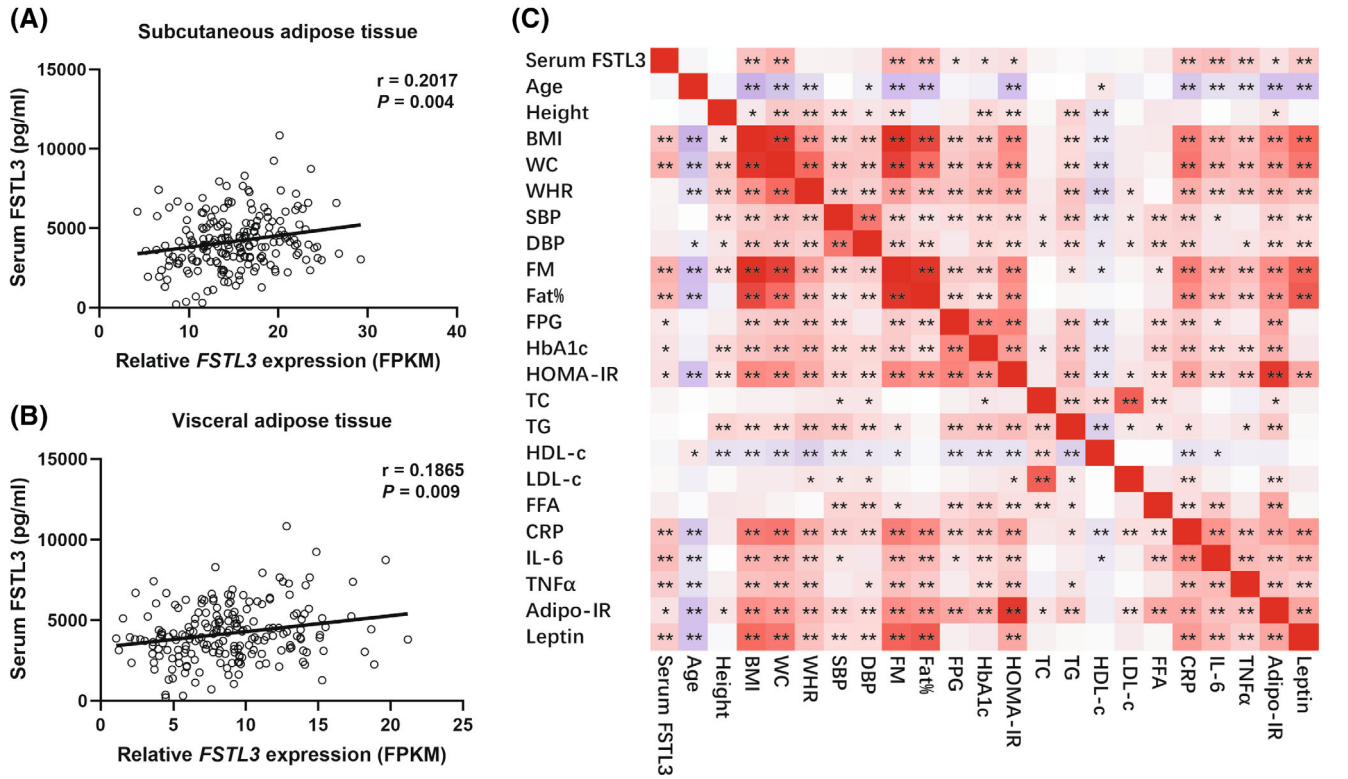

FIGURE 6 Relationship between serum FSTL3 and obesity-related indices. (A, B) Spearman analysis between serum FSTL3 levels and FSTL3 expression in subcutaneous or visceral adipose tissue. (C) Spearman analysis between serum FSTL3 levels and clinical indices. $^{*}p < 0.05$, $^{**}p < 0.01$. FPKM, fragments per kilobase of exon per million reads mapped; FSTL3, follistatin-like 3; WHR, waist-hip ratio [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Multivariate regression analyses showing factors independently associated with FSTL3

Variable	Standardized β	*t*	*p*
VFA	0.364	4.392	<0.001
TNFα	0.165	1.958	0.049

Note: Independent variables originally included: age, gender, smoking status, BMI, waist circumference, fat mass, homeostasis model assessment of insulin resistance, C-reactive protein, IL-6, TNFα, subcutaneous fat area, VFA.
Abbreviations: FSTL3, follistatin-like 3; VFA, visceral fat area.

myostatin expression both led to reduction of adipose tissue mass [30, 31]. However, the metabolic effect and the underlying mechanisms remain controversial and require further investigation. Our genome-wide coexpression analysis linked FSTL3 to inflammatory signaling pathways in human adipose tissue. Consistent with the analysis, we detected increased FSTL3 secretion when human primary adipocytes and adipose tissue were treated with TNFα, indicating that adipocyte FSTL3 secretion was regulated by inflammatory factors. One possible explanation was that the FSTL3 gene promoter contained an NF-κB-binding site [32]. Furthermore, we also found that recombinant FSTL3 could inhibit inflammatory gene expression regardless of the presence of TNFα. FSTL3 knockdown further increased the inflammatory gene expression and secretion with or without TNFα stimulation. It was reported that IL-1β was a proinflammatory cytokine related to adipocyte dysfunction [33], whereas CCL2 and IL-6 were known to contribute to macrophage infiltration and inflammation in adipose tissue [34, 35]. These results suggested that FSTL3 participated in regulating inflammatory responses, consistent with the phenotype of improved insulin sensitivity in FSTL3-overexpressing mice on a high-fat diet [8]. Considering our results, increased FM and inflammatory factors worked together to promote the expression and secretion of FSTL3 among individuals with overweight or obesity. In turn, increased FSTL3 could inhibit inflammatory progression. A previous study also reported that human plasma FSTL3 levels increased largely in response to a lipopolysaccharide bolus and infusion of TNFα [4], which was in agreement with our results in human primary adipocytes.

It remains to be elucidated how FSTL3 inhibits inflammation. FSTL3 shares structural and functional similarities with follistatin and it is able to neutralize activin and myostatin, which belong to the TGF-β superfamily [9]. A previous study reported that activin A could induce inflammatory responses through activating toll-like receptor 4 and the myeloid differentiation primary response protein MyD88 pathway and that follistatin administration altered the pattern of inflammatory cytokine release [36]. Another study showed that pharmacological inhibition of myostatin suppressed systemic inflammation in mice with chronic kidney disease [37]. Therefore, the anti-inflammatory role of FSTL3 might be mediated by

the downregulation of activin or myostatin. Alternatively, it was previously reported that inhibition of FSTL3 caused renal cell apoptosis via the glycogen synthase kinase 3 beta (GSK-3β) signaling pathway [38], and GSK-3β was reported to be involved in inflammation and endoplasmic reticulum stress [39]. Overall, these possible mechanisms remain to be further examined.

The consistency between FSTL3 expression and serum FSTL3 levels in adipose tissue was identified in our study; in addition to investigating the expression and function of FSTL3, we also analyzed the relationship between serum FSTL3 levels and clinical indicators. Clinical studies from Denmark and UK showed that plasma FSTL3 levels were associated with obesity-related indicators, such as age, BMI, and FM [4, 6]. Consistently, we found that serum FSTL3 levels were positively correlated with BMI, WC, FM, and fat%. Previous studies suggested that serum FSTL3 levels were associated with inflammatory factors [4] and involved in inflammatory responses during nonalcoholic steatohepatitis [40] and atherosclerosis progression [41]. The positively correlation between serum FSTL3 levels and inflammatory factors existed in our population, especially the independently positive correlation with TNFα. Our results also identified the relationship between inflammation and FSTL3 by ruling out smoking status as a confounder. In addition to inflammation, VAT accumulation was a risk factor for mortality independent of simple anthropometric indices [42]. Among Chinese individuals with overweight or obesity, VFA, rather than SFA, was independently positively correlated with serum FSTL3 levels after adjusting for confounding factors. The advantage of our study was the use of precise body fat parameters, which further clarified the association between VAT accumulation and serum FSTL3 levels. Therefore, FSTL3 accurately identified the obesity state with inflammation and VAT distribution, which could identify patients at increased risk for metabolic disorder progression.

There were several limitations in the present study. First, we did not investigate the mechanism by which FSTL3 regulates inflammation. Because insulin signaling was also enriched in our coexpression analysis, further research is needed to clarify other potential regulatory factors of adipocyte FSTL3 secretion. Secondly, this study has a relatively small serum sample size, which made it difficult to clarify the causal relationship between increased serum FSTL3 levels and visceral fat accumulation. Thirdly, we did not collect detailed dietary intake and exercise, which could not rule out possible effects of dietary intake and exercise as confounders in this study. In addition, 2-hour postprandial plasma glucose was not measured in the normal-weight individuals, so the present study would have excluded potential cases of diabetes though combining FPG and HbA_{1c} is associated with a lower rate of missed diabetes diagnosis [43]. Hence, further prospective studies are warranted to confirm the present findings in a larger and prospective population.

CONCLUSION

Taken together, we first demonstrated that FSTL3 was secreted by human mature adipocytes and participated in the adipocyte inflammatory response. Serum FSTL3 levels were independently and positively related to visceral fat accumulation and inflammation among Chinese individuals with overweight or obesity.

AUTHOR CONTRIBUTIONS

YB and XL designed research. XL and HY carried out the experiments and collected clinical data. YW performed the serum FSTL3 measurements. XL and HZ performed the statistical analysis and wrote the paper. YB, YY, and XM revised the paper and contributed to the discussion. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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