GREM2 is associated with human central obesity and inhibits visceral preadipocyte browning

Wen Liu,a,b,1 Danjie Li,a,1 Minglan Yang,a,2 Long Wang,a,2 Yu Xu,a,1 Na Chen,a,1 Zhiyin Zhang,a,1 Juan Shi,a,1 Wen Li,a,1 Shaqian Zhao,a,1 Aibo Gao,a,1 Yufei Chen,a,1 Qinyun Ma,a,1 Ruizhi Zheng,a,1 Shujing Wang,a,2 Yifei Zhang,a,1 Yuhong Chen,a,1 Shuwen Qian,a,1 Yufang Bi,a,1 Weiqiong Gu,a,1 Qiqun Tang,c Guang Ning,a,b,1 Ruixin Liu,a,1 Weiqing Wang,a,1 Jie Hong,a,1 and Jiqiu Wanga,b,*

aDepartment of Endocrine and Metabolic Diseases, Shanghai Institute of Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
bShanghai National Clinical Research Center for Metabolic Diseases, Key Laboratory for Endocrine and Metabolic Diseases of the National Health Commission of the PR China, Shanghai Key Laboratory for Endocrine Tumor, State Key Laboratory of Medical Genomics, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
cThe Key Laboratory of Metabolism and Molecular Medicine of the Ministry of Education, Department of Biochemistry and Molecular Biology of School of Basic Medical Sciences, Fudan University Shanghai Medical College, Shanghai, China

Summary

Background Some circulating proteins are linked to central adiposity. Gremlin 2 (GREM2) functions as a secreted factor involved in osteogenesis and adipogenesis. Here, we investigated the association of blood GREM2 levels and central adiposity, and the biological roles of GREM2 in the browning program of visceral preadipocytes.

Methods Three independent cohorts were applied to detect circulating GREM2 levels. Recombinant Grem2 protein, Grem2 overexpression and knockout mouse models, and preadipocyte-specific Bmpr2 knockout mice were used to assess the roles of Grem2 in the browning program.

Findings We detected the presence of GREM2 protein in human serum using an ELISA approach. We revealed elevated GREM2 levels in severely obese subjects and validated this finding in a large-scale community population involving 10,327 subjects. Notably, serum GREM2 was positively associated with visceral fat volume, as quantified by 3D reconstruction methods. In mice, Grem2 was highly expressed in visceral fat and liver tissues, while surgical removal of visceral fat lowered circulating Grem2 levels. Visceral fat secreted more Grem2 in obese mice. Grem2-overexpressed mice exhibited a reduced browning ability of visceral fat, whereas Grem2 ablation enhanced the browning capacity and reduced visceral fat content. Mechanistically, Grem2 attenuated the browning program of visceral preadipocytes partially by antagonizing BMP4/7-SMAD1/5/8 signaling pathway. Further, genetic deletion of Bmpr2 in Pdgfra+ preadipocytes abolished the antagonistic effect of Grem2.

Interpretation These findings indicate that GREM2 might function as a circulating protein factor associated with human visceral adiposity, and Grem2 inhibits the browning capacity of visceral preadipocytes partially by BMP4/7-BMPR2 signaling pathway.

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Keywords: GREM2; Serum biomarker; Central obesity; Visceral fat; Browning

Introduction

Obesity has become a growing epidemic worldwide in recent decades and is associated with a series of leading causes of death.1,2 Unlike general obesity, central obesity is characterized by excessive visceral fat accumulation, which is particularly vulnerable to lipid overload and is recognized as a driver of cardiometabolic diseases.3,4 One challenge in combating central obesity is that there are few effective and safe strategies to lose visceral fat once it has been deposited.5 Although the reasons for depot-specific fat deposition remain unclear, visceral fat accumulation is partially attributed to the reduced mitochondrial respiratory function and oxidative phosphorylation of visceral adipocytes when compared with subcutaneous adipocytes.6

*Corresponding author.
E-mail address: wangjq@shsmu.edu.cn (J. Wang).
1 These authors contribute equally to this work.
GREM2 is a secreted protein and functions to antagonize the biological roles of BMP2/4 in regulating osteogenesis of stromal cells and adipogenesis of preadipocytes. Visceral fat has a weak browning capacity to some extent attributed to impairment of BMPs-SMADs signaling. Excess visceral fat deposit is tightly associated with malignant metabolic consequences, e.g., type 2 diabetes and cardiovascular diseases. Therefore, this study aims to investigate whether circulating GREM2 levels is associated with human visceral adiposity, and how GREM2 regulates the browning program of visceral preadipocytes.

This study reports that circulating GREM2 levels are positively associated with visceral obesity in humans, and are especially higher in normal-weight subjects with central obesity. In mice, Grem2 is highly expressed in visceral fat and its expression changes in response to external cues like coldness and fasting-refeeding. Visceral fat of ob/ob mice secreted more Grem2. Overexpression of Grem2 attenuates whereas ablation of Grem2 enhances the browning abilities of visceral fat. Moreover, Grem2 inhibits the browning adipocyte differentiation partially by antagonizing BMP4/7-BMPR2-SMADs signaling in preadipocytes.

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GREM2, which was previously named PRDC (Protein Related to DAN and Cerberus) and is recognized as a secreted protein, belongs to the differential screening-selected gene aberrative in the neuroblastoma (DAN) family and can bind to certain BMPs with high affinity, acting as an extracellular BMP antagonist. In our previous study, the GREM2 signal was initially screened out from the expression profile of visceral WAT in Lgr4-deficient mice (GSE195570), which exhibited marked visceral browning and obesity-resistant phenotypes. We hypothesized that GREM2 might be involved in the browning process and obesity development. We first found that GREM2 can be detected in the circulating system, and its levels were associated with central obesity in three independent Chinese cohorts. Furthermore, we investigated the biological roles and potential mechanism of GREM2 in the browning process of visceral (pre)adipocytes both in vitro and in vivo. This study may provide us a circulating biomarker for visceral adiposity and a potential target for clinical intervention of central obesity.

Brown adipocytes have a high mitochondria content and powerful capacity to metabolize fatty acids into heat. Emerging evidence has shown that brown-like (beige) adipocytes can originate from progenitors or preadipocytes in stromal vascular fractions (SVFs) of subcutaneous white adipose tissue (sWAT), which functionally and morphologically resembles classical brown adipocytes and has the potential to treat obesity. In contrast, visceral white adipose tissue (vWAT) located deeper in the abdominal cavity shows resistance to browning inducers. Visfatin, which is highly secreted from visceral fat both in humans and mice, was once reported to be correlated with visceral adiposity and exerted insulin-mimetic effects, but these findings has not been well validated by subsequent studies. Theoretically, identifying unknown adipokines associated vWAT may provide a potential way to stratify obesity or even reduce central adiposity.

Among the secreted factors from adipose tissues, decreased activities of the transforming growth factor-β (TGF-β)/bone morphogenetic protein (BMP) signaling pathways are considered to involve in fat accumulation and obesity development. As two attractive BMPs, BMP4 mediates the browning of white adipose tissues (WATs) to improve glucose and energy homeostasis, while BMP7 acts as a key regulator of classical brown adipose tissue (BAT) development. In comparison with BMP members, few studies have investigated the clinical implications and biological roles of BMP antagonists in metabolic disorders, among which Gremlin 2 (GREM2) was reported to increase in adipose tissues with aging and regulate the adipogenesis and osteogenesis. However, the effects of GREM2 on the browning program and central obesity are poorly understood.

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**Methods**

**Human participants**

In this study, three independent cohorts were included to evaluate the association between circulating GREM2 levels and adiposity. In the discovery stage, the Genetics of Obesity in Chinese Youngs (GOCY) study that recruited young, severely obese patients was applied. The detailed information of this cohort was described previously and registered in ClinicalTrials.gov (Clinical trial reg. no. NCT01084967, https://clinicaltrials.gov). In the validation stage, we utilized a general population including 10,327 participants from a community-based health survey in Jiading District, Shanghai. The third cohort was from a deep phenotyping study, which was designed as a longitudinal follow-up of metabolic diseases at SAIC Volkswagen Automotive Company Limited, Shanghai (MedSV...
In the third cohort, we further performed visceral fat mass estimation using three-dimensional (3D) computed tomography (CT) reconstruction in 376 volunteers. Cross-sectional subcutaneous and visceral fat areas were measured at the umbilical level and calculated using FatScan software (N2 Systems Inc.). The volume of subcutaneous and visceral fat was calculated as volume = number of voxels \times\text{ voxel size}, where voxel size was obtained from Digital Imaging and Communications in Medicine (DICOM) information from corresponding CT images. In all cohorts, anthropometric examinations were performed according to standard protocols, and circulating GREM2 levels were determined using a commercial Grem2 ELISA kit (dy2069, R&D) according to the manufacturer’s protocol.

Mice
In the epididymal white adipose tissue (eWAT)-surgical removal experiment, eight male C57BL/6 mice had their epididymal fat pads on both sides excised completely. Eight sham-treated mice underwent the same surgical procedure without fat pad excision. The mice were euthanized for blood collection 10 days after surgery. In the cold stress experiments, mice were subjected to cold room stress (6°C) for 24 h or 10 days before sample collection. Global Grem2 transgenic and Grem2\text{lox}{\text{p}}/\text{lox}{\text{p}} mice were first generated by a commercial provider (Cyagen) (see supplementary figures). Global Grem2 transgenic, Grem2\text{lox}{\text{p}}/\text{lox}{\text{p}};\text{P}2\text{-cre} mice and their littermates were subjected to cold room stress (6°C) for 10 days before euthanasia. Tissue samples were collected from animals after euthanasia and stored in a −80°C freezer or in 4% paraformaldehyde (PFA) until use. Preadipocyte Bmp2 knockout mice were generated by crossing Bmp2\text{lox}{\text{p}}/\text{lox}{\text{p}} mice with Pdgfra-cre (JAX stock No. 013148) mice. Homozygous flox with cre-negative mice was used as controls. The mice were maintained on a 12h:12h light-dark cycle with free access to water and normal chow diet (NCD). A metabolic cage (Columbus Instruments) was used to evaluate the energy expenditure and food intake. 19\text{ ob/ob} (B6/JfGpt-Ltp\text{nmcd}20/Gpt, stock no. T001461, RRID: IMSR_GPT:T001461) and wild-type (WT) (n = 7–7) littermates were obtained from GemPharmatech. Sample sizes were estimated from previous experience and routine protocols of animal studies, and no additional statistical method was used to predetermine sample size. No randomization was performed during the experiment. Investigators were not blinded when carrying out the experiment, but were blinded during the allocation and sample collection. Inclusion/exclusion criteria is pre-established before the experiments. Mice were placed in the same environment and were measured/treated in an alternating order to minimize the confounders that may impact the outcome of the experiments.

Intraperitoneal glucose tolerance test (IPGTT)
Mice were fasted overnight for 16 h and injected with D-glucose (2 g/kg) intraperitoneally for the glucose tolerance test. Mouse blood glucose levels were measured with whole blood from the tail vein using a glucose meter (LifeScan).

The purification procedures of recombinant mouse Grem2 protein
Pet12b-mGrem2/BL21 (DE3) was inoculated in LB medium (plus 100 microns/ml ampicillin) for 3.5 h. The bacterial cells were collected and centrifuged at 6000 \times \text{ g} for 10 min at 4°C, followed by resuspension in lysis buffer and ultrasonic crushing. The inclusion bodies were washed by ultrasound treatment and dissolved in the buffer, followed by the application of Superdex 200 (GE Healthcare) molecular sieve chromatography and sulfopropyl (SP) cation exchange chromatography, which was used to collect the mouse Grem2 (mGrem2) components. Next, mGrem2 obtained by SP cation exchange chromatography was renaturated and refined by Mono S cation exchange chromatography and Superdex 75 (GE Healthcare) molecular sieve to remove polymers and obtain pure mGREM2. The purified mGREM2 was concentrated to 1-6 mg/mL using a 10-KD ultrafiltration concentration tube and stored at −80°C. Detailed information is provided in Supplementary Figure 1.

Cell culture and plasmid transfection
Human embryonic kidney 293T (HEK293T) cells (ATCC, RRID: CVCL_1926) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Cat#10313021) containing 10% fetal bovine serum (FBS) (Gibco, Cat#10270-106) at 37°C in the presence of 5% CO2. HEK293T cells were seeded in 24-well plates and transfected with 1-0 μg of pcDNA3.1 vector, vector-expressing plasmid containing the mouse Grem2 (Reference Sequence: NM_011825.1) and human GREM2 (Reference Sequence: NM_022469.4) plasmids, respectively. For co-immunoprecipitation, expression constructs encoding Myc-tagged (C-terminal) human BMP4 or BMP7, and Flag-tagged (C-terminal) mouse Grem2 cDNAs in the pCMV-Entry expression vector were acquired from Xitu company (NM_001202 for BMP4, NM_001719 for BMP7, and NM_011825 for Grem2) and were verified by Sanger sequencing. The plasmids were transfected into HEK293T cells with the Lipofectamine 2000 (Invitrogen, Cat#11668019). Mouse and human recombinant GREM2 proteins used for the evaluation of ELISA kit were purchased from R&D.
Grem2 secretion by visceral fat
In brief, intact unilateral epididymal fat depots were isolated from 8-week-old male ob/ob and WT mice, respectively (n = 77). Every fat pad was then cut into several pieces, placed into a 6-well plate, and covered with a 70 µm strainer to maintain its fully submergence in the culture medium of 6 ml DMEM/F12 (Gibco, Cat#11320082) supplemented with 10% FBS (plus 1% penicillin/streptomycin and 1 mM L-glutamine, Gibco, Cat#15140122, 25030-081). Fat tissues were incubated for 6 h, and the condition media were collected at each time point (1 h, 3 h, and 6 h, respectively).

Stromal vascular fractions (SVFs) isolation and beige adipocyte differentiation
For beige adipocyte induction, the visceral WAT-derived SVFs of humans, male 129S1 mice (Charles River Laboratories), Grem2floxp/floxp, Grem2flopxflopx;Pdgfra-cre, or Bmpr2flopxflopx;Pdgfra-cre mice and their littermate controls were first isolated and then seeded onto 12- or 24-well plates to reach confluence before induction. Beige and white adipocyte induction cocktails were used as previously described.19 For beige adipocyte induction cocktail, SVFs were initiated to induction with growth medium supplemented with 8.4 µg/ml Insulin, 0.5 mM Isobutylmethylxanthine (IBMX) (Sigma), 1 µM Dexamethasone (DEX) (Sigma), 1 mM Triiodothyronine (T3) (Sigma) and 1 µM Roglitazone (Sigma) for 4 days, and subsequently in growth medium supplemented with 8.4 µg/ml Insulin, 1 mM T3 and 5 µM Roglitazone for the following 4 days. For white adipocyte cocktail, SVFs were induced with growth medium supplemented with 8.4 µg/ml Insulin, 0.5 mM IBMX, 1 µM DEX for 2 days, and in growth medium supplemented with 8.4 µg/ml insulin for the following 6 days. To evaluate the effects of Grem2 on the browning process, recombinant mouse Grem2 protein was added twice (on days -3 and -1, respectively) before the induction procedure. Recombinant human BMP4 or BMP7 proteins (R&D, Cat#MAB757-100, MAB3541-100) were combined with Grem2 in the antagonizing experiments.

Oxygen consumption rate (OCR) measurement
OCRs of differentiated beige adipocytes derived from visceral SVFs were measured at 37 °C using an XF24 analyzer (Seahorse Bioscience) according to the manufacturer's instructions. Oligomycin (1 µM), carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (2 µM), and rotenone/antimycin (1 µM/1 µM) were used to detect basal, uncoupled, maximal, and non-mitochondrial respiration, respectively.

Western blotting assay
Proteins were extracted using the RIPA (Beyotime Biotechnology, Cat#R20095) reagent. All protein concentrations were determined using the BCA protein assay (Thermo fisher, Cat#23227), followed by western blotting as previously described.19 The resulting bands were visualized using the Imagine QAS 4000 imaging system (GE Healthcare). Detailed information regarding the antibodies is provided in Supplementary Table 1.

Real-time PCR
Total RNA was prepared using the TRIzol reagent (Invitrogen, Cat#15596-018). Real-time PCR was performed with a Quant Studio Real-time PCR Instrument (Applied Biosystems) using SYBR Green Supermix (Takara, Cat#RR420A). Data were normalized to 36B4 expression and analyzed using the 2−∆∆CT method. Detailed primer information is provided in Supplementary Table 2.

Co-immunoprecipitation (co-IP) assay
HEK293T cells were transfected with Myc-tagged BMP4 (or BMP7) and Flag-tagged Grem2 plasmids, and then were collected 24 h later with a NP40 lysis buffer (Beyotime, Cat#P0013F) containing a phosphatase inhibitor cocktail (Roche, Cat#11836153001). After cell lysate was centrifuged at maximum speed, 10% input was taken from the supernatant, and the remaining lysate was incubated with 10 µL Anti-c-Myc magnetic beads (MCE, Cat#HY-K0206) on a rotator overnight at 4 °C. At last, the immunoprecipitate was washed with NP40 for 5 times, and boiled in 2x Laemmlli Sample Buffer (BioRad) at 99 °C for 10 min for western blotting analysis.

Staining studies
The staining of Oil Red O (Nanjing Jiancheng Bioengineering Institute) of induced mature beige adipocytes, hematoxylin and eosin (H&E) staining, and immunohistochemical (IHC) staining of uncoupled protein 1 (UCP1) in epididymal fat were performed as previously described,19 and images were captured using a microscope (Nikon Eclipse Ci-L).

Statistics
In human studies, the distributions of continuous variables were examined by their cumulative frequency distribution charts (proc gchart in SAS). Metabolic indicators, such as body mass index (BMI), waist circumference (WC), waist-height ratio (WHr), waist-hip ratio (WHr), fasting blood glucose (FBG), HbA1c, total cholesterol, LDL-c, HDL-c, visceral fat area (VFA), visceral fat volume (VVF), and visceral fat content (VFC), presented normal distributions and were presented as the mean ± standard deviation (SD). Other metabolic indicators, such as serum GREM2, triglycerides, fasting insulin, and HOMA-IR, presented non-normally
distribut ed and presented as median (interquartile range).

To achieve normality, these non-normally distributed values were log-transformed (ln) before analysis. Categorical variables were presented as numbers with proportions.

Parameters (Figure 1c,d,h) and Supplementary Table 3) were adjusted in GOCY study as noted (Table 1, Figure 1b) or one-way analysis of variance (ANOVA) (Figure 1a, Supplementary Tables 4 and 6).

Pearson correlation analysis was used to explore the correlation between serum lnGREM2 levels and metabolic parameters (Figure 1c,d,h–j) and Supplementary Fig. 3). Three logistic regression models were built to investigate the risk of prevalent general obesity and central obesity in relation to each quartile increase in serum GREM2 or each 1-SD increase in lnGREM2. In model 1, no covariates were adjusted. In model 2, age and sex were adjusted. In model 3, age, sex, education level, current alcohol consumption, current smoking habit, physical activity level, diabetes, hypertension, and dyslipidemia status were all adjusted in GOCY study as noted (Table 1, Figure 1b) or one-way analysis of variance (ANOVA) (Figure 1a, Supplementary Tables 4 and 6). Pearson correlation analysis was used to explore the correlation between serum lnGREM2 levels and metabolic parameters (Figure 1c,d,h–j) and Supplementary Fig. 3). Three logistic regression models were built to investigate the risk of prevalent general obesity and central obesity in relation to each quartile increase in serum GREM2 or each 1-SD increase in lnGREM2. In model 1, no covariates were adjusted. In model 2, age and sex were adjusted. In model 3, age, sex, education level, current alcohol consumption, current smoking habit, physical activity level, diabetes, hypertension, and dyslipidemia status were all adjusted in GOCY study as noted (Table 1, Figure 1b) or one-way analysis of variance (ANOVA) (Figure 1a, Supplementary Tables 4 and 6). Pearson correlation analysis was used to explore the correlation between serum lnGREM2 levels and metabolic parameters (Figure 1c,d,h–j) and Supplementary Fig. 3). Three logistic regression models were built to investigate the risk of prevalent general obesity and central obesity in relation to each quartile increase in serum GREM2 or each 1-SD increase in lnGREM2. In model 1, no covariates were adjusted. In model 2, age and sex were adjusted. In model 3, age, sex, education level, current alcohol consumption, current smoking habit, physical activity level, diabetes, hypertension, and dyslipidemia status were all adjusted in GOCY study as noted (Table 1, Figure 1b) or one-way analysis of variance (ANOVA) (Figure 1a, Supplementary Tables 4 and 6).

### Table 1: The clinical characteristics of young, severely obese subjects and lean controls in GOCY study (n = 310).

|                      | Controls                  | Obesity                  | Diabetes                  | P value<sup>1</sup> | P value<sup>2</sup> |
|----------------------|---------------------------|--------------------------|---------------------------|----------------------|----------------------|
|                      | All non-diabetes          | Non-diabetes             | Diabetes                  |                      |                      |
| Age (years)          | 23.39 ± 1.86              | 23.19 ± 4.35             | 25.59 ± 6.03              | 0.9049               | 0.0410               |
| Sex (men)            | 146 (52)                  | 147 (57)                 | 17 (8)                    | 0.4664               | 0.5086               |
| BMI (kg/m<sup>2</sup>)| 20.18 ± 1.19              | 36.70 ± 5.29             | 38.22 ± 4.39              | <0.0001              | <0.0001              |
| WC (cm)              | 71.56 ± 5.76              | 112.48 ± 12.46           | 115.39 ± 8.28             | <0.0001              | <0.0001              |
| WHR                  | 0.43 ± 0.03               | 0.67 ± 0.07              | 0.69 ± 0.06               | <0.0001              | <0.0001              |
| WHR                  | 0.78 ± 0.05               | 0.95 ± 0.07              | 0.98 ± 0.05               | <0.0001              | <0.0001              |
| FBG (mmol/l)         | 4.78 ± 0.34               | 5.18 ± 0.54              | 7.46 ± 1.44               | <0.0001              | <0.0001              |
| Fasting insulin (IU/ml) | 6.16 (4.86–8.09)         | 21.86 (17.52–30.39)      | 26.55 (19.50–30.90)       | <0.0001              | <0.0001              |
| HDMA-IR              | 1.30 (1.00–1.73)          | 5.16 (3.82–7.13)         | 8.09 (7.41–8.85)          | <0.0001              | <0.0001              |
| HbA1C (%)            | 5.11 ± 0.24               | 5.74 ± 0.78              | 7.31 ± 1.95               | <0.0001              | <0.0001              |
| Triglycerides (mmol/l) | 0.68 (0.56–0.86)         | 1.51 (1.15–1.91)         | 1.86 (1.25–2.16)          | <0.0001              | <0.0001              |
| Total cholesterol (mmol/l) | 4.06 ± 0.68              | 4.53 ± 0.88              | 5.00 ± 1.21               | <0.0001              | <0.0001              |
| HDL-c (mmol/l)       | 1.51 ± 0.29               | 1.02 ± 0.24              | 0.97 ± 0.13               | <0.0001              | <0.0001              |
| LDL-c (mmol/l)       | 2.17 ± 0.54               | 2.82 ± 0.74              | 3.40 ± 0.75               | <0.0001              | <0.0001              |
| GREM2, pg/ml         | 924.18 (813.33–1068.65)   | 1102.71 (988.55–1273.38) | 1303.51 (997.42–1467.39)  | <0.0001              | <0.0001              |

Note: Variables with normal distribution data are presented as mean ± SD; Variables with skewed distribution data are presented as median with inter-quartile range. BMI, body mass index; WC, waist circumference; WHR, waist-height ratio; WHR, waist-hip ratio; FBG, fasting blood glucose; HOMA-IR, homeostasis model assessment for insulin resistance; HDMA-IR = fasting insulin (IU/ml) × fasting glucose (mmol/l)/22.5; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. P value<sup>1</sup> indicated comparison between obese group and lean control group. P value<sup>2</sup> indicated comparison between non-diabetes and diabetes in obesity group.

### Ethics

The human studies were approved by the Institutional Review Board of the Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (SJTUM) (No. 2010-006) and were in accordance with the principle of the Helsinki Declaration (No. KY2016-75). Written informed consent was obtained from each participant.

Animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications No. 8023, revised 1978). All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of SJTUM (No. B-2018-005). SJTUM animal care and use program is accredited by the AAALAC International.

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The funders had no role in study design, data collection, data analysis, interpretation, writing of the manuscript, or in the decision to submit the paper for publication.

### Results

**Human GREM2 measurement with an ELISA method**

Human GREM2 (hGREM2) is highly conserved with its homolog, mouse Grem2 (mGrem2), showing 95.2% (160/168 amino acids) identity and 97.6% (164/168 amino acids) similarity (Supplementary Fig. 2a).
Therefore, a commercially available mGrem2 ELISA kit was tested to detect hGREM2 protein. After equivalent overexpression of two plasmids in HEK293T cells, mGrem2 and hGREM2 proteins in cell lysates showed comparable expression levels by using an anti-mGrem2 antibody, as well as through measurement by the ELISA kit (Supplementary Fig. 2b,c). Simultaneously, no signals in the vector group were detected, indicating a high specificity of the ELISA detection antibodies. As expected, mGrem2 and hGREM2 were successfully detected in the conditioned media of transfected cells (Supplementary Fig. 2d), indicating that GREM2 is a secreted protein. We also used the ELISA kit to measure commercially available recombinant mGrem2 and hGREM2 proteins and found a high consistency within a wide concentration range, showing a high measurement accuracy (Supplementary Fig. 2e). Two biological supernatant replicates of induced beige adipocytes derived from the SVFs of human visceral fat were used to measure the secreted hGREM2 protein in conditioned media in Figure 1.

Figure 1. Serum GREM2 levels are elevated in individuals with obesity and positively associated with central obesity. a. The increase trend of serum GREM2 in parallel with the increased BMI in GOCY study (n = 146 for lean controls; n = 164 for young, severely obese subjects). b. Serum GREM2 levels are higher in GOCY participants with central obesity (n = 144 for normal WC, n = 160 for central obesity defined by WC; WC data were not recorded intactly for 6 subjects). c-d. The correlation of lnGREM2 and BMI (c) and WC (d), respectively, in the validation population (n = 10,327). e. Mean levels of serum GREM2 (lnGREM2) were calculated by the population stratification according to BMI and WC cutoffs. F-g. Visceral fat pads were imaged by CT scanning, and the representative cross-section visceral fat area (VFA) (f) and reconstituted 3D visceral fat volume (VFV) (g) were shown, respectively. h-i. The correlation of serum lnGREM2 levels and VFA (n = 576) (h) and VFV (n = 576) (i) measurements, respectively. j. The correlation of serum lnGREM2 levels and visceral fat content (VFC) measured (n = 576). VFC, visceral fat volume (mL)/body weight (kg). Dose-response association of lnGREM2 with obesity parameters was constructed in linear regression, with shaded area indicating 95% CI. For a, one-way analysis of variance (ANOVA) was used for the multiple group comparisons. For b, two-tailed Student’s t-test was used for two group comparisons. *** p < 0.005.
respectively. Interestingly, higher serum GREM2 by WC is a common clinical parameter used to define central obesity. GREM2 levels was observed after adjusting for age and increase in BMI, a significant increasing trend in serum GREM2 levels was positively correlated with metabolic parameters, including FBG, fasting insulin, HOMA-IR, HbA1c, triglycerides, total cholesterol, and LDL-c, but were inversely correlated with HDL-c (Supplementary Fig. 3). We then divided all obese individuals into three subgroups according to their BMI levels. In parallel with the increase in BMI, a significant increasing trend in GREM2 levels was observed after adjusting for age and sex (P < 0.0001) (Figure 1a). Waist circumference (WC) is a common clinical parameter used to define central obesity by WC ≥ 90 cm (men) and 85 cm (women), respectively. Interestingly, higher serum GREM2 levels were observed in subjects with central obesity defined by the WC cutoffs after adjusting for age and sex (P < 0.0001) (Figure 1b). Next, using binary logistic regression analysis, we found that serum GREM2 levels were positively correlated with general obesity defined by BMI ≥ 30.0 kg/m² (odds ratio [OR]: 1.76, 95% confidence interval [CI]: 1.13–2.75, P = 0.013), as well as for central obesity, respectively, defined by the above WC cutoffs (OR: 1.66, 95% CI: 1.07–2.58, P = 0.023) or by waist-height ratio (WHR) ≥ 0.51 (men) and 0.52 (women) (OR: 1.55, 95% CI: 0.11–3.25, P = 0.047), but not significant by waist-hip ratio (WHR) ≥ 0.85 (men) and 0.80 (women) (OR: 1.27, 95% CI: 0.87–1.85, P = 0.214), after adjusting for potential confounders (Supplementary Table 3). This case-control study suggests that secreted GREM2 protein can be detected in human serum, and serum GREM2 levels are positively associated with general and central obesity.

| Quartile 1 | Quartile 2 | Quartile 3 | Quartile 4 | P for trend |
|-----------|-----------|-----------|-----------|-------------|
| General obesity | Higher WC | Higher WHtR | Higher WHR | General obesity |
| 1.00 | 1.21 (0.97–1.52) | 1.26 (1.13–1.41) | 1.29 (1.15–1.46) | 1.19 (1.02–1.38) | 1.48 (1.31–1.67) | <0.0001 |
| 1.00 | 1.25 (1.00–1.56) | 1.40 (1.25–1.56) | 1.40 (1.24–1.58) | 1.25 (1.07–1.45) | 1.47 (1.18–1.82) | <0.0001 |
| 1.00 | 1.32 (1.18–1.47) | 1.38 (1.22–1.52) | 1.47 (1.31–1.65) | 1.32 (1.18–1.47) | 1.36 (1.22–1.52) | 0.0008 |
| 1.00 | 1.41 (1.34–1.40) | 1.47 (1.37–1.56) | 1.47 (1.31–1.65) | 1.41 (1.34–1.40) | 1.47 (1.31–1.65) | <0.0001 |
| 1.00 | 1.46 (1.34–1.40) | 1.47 (1.37–1.56) | 1.47 (1.31–1.65) | 1.46 (1.34–1.40) | 1.47 (1.37–1.56) | <0.0001 |

Table 2: The risk of general and central obesity in relation to quartiles of serum GREM2 levels in a large-scale population cohort (n = 10,327).

Note:
Model 1 was unadjusted.
Model 2 was adjusted for age and sex.
Model 3 was adjusted for age, sex, education level, current alcohol consumption, current smoking habit, physical activity level, and diabetes, hypertension, and dyslipidemia status.

Large-scale population study validates the positive association between serum GREM2 and central obesity

In the validation stage, we further measured serum GREM2 levels in a large-scale population including 10,327 participants. General characteristics according to the quartiles of serum GREM2 levels are summarized in Supplementary Table 4. The prevalence of general obesity (defined by BMI) and central obesity (defined by WC, WHtR, or WHR) gradually increased as GREM2 levels increased (P < 0.0001 for all trends, Supplementary Table 4). Furthermore, serum GREM2 levels were positively correlated with BMI and WC in
the total population (Figure 1c,d). With the lowest quartile of serum GREM2 levels as the reference group, univariate logistic regression analysis showed significantly increased risks for general obesity, higher WC, higher WHtR, and higher WHR (Table 2). The association remained significant after adjustment for age and sex or after further adjustment for other potentially confounding factors (Table 2).

Furthermore, using GREM2 level as a continuous variable, the following significant ORs associated with a 1-SD increase of lnGREM2 levels were observed: 1.08 (95% CI, 1.04–1.13) for higher WC, 1.07 (95% CI, 1.03–1.11) for higher WHtR, and 1.05 (95% CI, 1.01–1.09) for higher WHR, after full adjustment for the covariates. A non-significant OR of 1.05 (95% CI, 1.00–1.10) for general obesity (BMI) was associated with a 1-SD increase of lnGREM2 levels (Supplementary Table 5). We then stratified the population and found the strongest association between lnGREM2 levels and WC in subjects with BMI ≤ 24.0 kg/m² (OR, 1.27; 95% CI, 1.04–1.54), compared with subjects with BMI > 24.0 kg/m² (OR, 1.05; 95% CI, 1.00–1.11) (P for interaction < 0.0001, Supplementary Fig. 4). Next, we stratified all participants based on BMI and WC cutoffs. In each BMI category (BMI ≤ 24.0, 24.0–28.0 or ≥ 28.0 kg/m²), according to the overweight/obesity criteria for China,34 subjects with higher WC had higher GREM2 levels; whereas, in the subgroup with central obesity, serum GREM2 levels showed a decreasing trend in three BMI categories (Figure 1e). In this context, subjects with normal BMI (≤ 24.0 kg/m²) but central obesity (WC ≥ 90 cm [men] or ≥ 85 cm [women]) had the highest GREM2 levels in all six subgroups (Figure 1e). Together, these data indicate that serum GREM2 levels are more tightly associated with central obesity and relatively weakly associated with general obesity.

Elevated serum GREM2 levels and visceral fat deposits in humans

Excess fat accumulation in the abdominal cavity is a core feature of central obesity.35,36 To evaluate the potential linkage between visceral fat content and serum GREM2 levels, the third independent cohort with 192 lean controls (mean BMI ± SD, 21.48 ± 1.81 kg/m²), 134 overweight (25.87 ± 1.15 kg/m²) and 250 obese individuals (32.08 ± 2.66 kg/m²) (Supplementary Table 6) received an abdominal-pelvic CT scan.24 We calculated the visceral fat areas (VFAs) at the umbilical level (Figure 1f) using FatScan software40,41 and whole visceral fat volume (VVF) using precise 3D CT reconstruction (Figure 1g) and defined a new obesity index, visceral fat content (VFC = VVF/body weight).24 Of importance, we further confirmed the positive association between serum GREM2 levels and VFA, VVF, and VFC, respectively (Figure 1h–j). This cohort study suggests that serum GREM2 levels are significantly associated with visceral fat deposits.

More GREM2 is secreted by visceral fat in adiposity status and its local expression is repressed during the browning process

Among various tissues of 8-week-old male C57BL/6J mice, eWAT (referred as classical visceral fat in mice) showed an abundant Grem2 mRNA expression (Figure 2a). After surgical removal of all eWAT pads, we detected a modest decrease in plasma Grem2 compared with sham-treated mice, suggesting a contribution of visceral fat to circulating Grem2 (Figure 2b). Physiologically, short-term fasting mobilizes visceral WAT more profoundly than subcutaneous WAT.35 A marginal decrease of plasma Grem2 after 24 h fasting followed by a pronounced rebound after 24 h refeeding (Figure 2c). Furthermore, in ob/ob mice, GREM2 was elevated in the circulating system and eWAT (Figure 2d,e). Of importance, when the visceral fat pads isolated from ob/ob and WT mice were incubated ex vivo, ob/ob visceral fat secreted more Grem2 into extracellular fluids (Figure 2f,g). Interestingly, plasma GREM2 levels slightly declined following browning stimulation by short- and long-term cold stress in mice (Figure 2h). In consistence, under cold stress, a moderate reduction of Grem2 protein levels was detected in eWAT but not in inguinal white adipose tissue (iWAT) or BAT (Figure 2i). We thus isolated eWAT-derived SVFs and mature white adipocytes (MAs) from NCD and high-fat diet (HFD) mice, respectively, and found that Grem2 mRNA was preferentially expressed in MAs relative to SVFs (Figure 2j). Once primary SVFs were induced into mature beige adipocytes, a reduction in Grem2 mRNA expression and secreted Grem2 proteins were observed (Figure 2k,l). Together, these physiological and interventional results suggest that visceral fat releases more Grem2 in obese status, the circulating GREM2 levels fluctuate with visceral mass changes, and Grem2 might be involved in the browning process of visceral WAT.

Grem2 attenuates the browning capacity of visceral fat

A relatively weak browning capacity of visceral preadipocytes has been well described, when compared with subcutaneous preadipocytes.35 We then pre-treated eWAT-derived SVFs of recombinant Grem2 protein only for 3 days before beige-adipocyte induction (Figure 3a). Despite with a low basal level, the mRNA and protein expression of uncoupled protein 1 (Ucp1), a brown/beige adipocyte marker, were detectable and could be inhibited by Grem2 in a concentration-dependent manner in differentiated mature beige adipocytes (Figure 3b,c). The expression of other BAT-related genes, including Dio2 and C/ebpβ, was also reduced by
high-concentration Grem2 treatment (Figure 3b). However, when the SVFs were induced with white-adipocyte induction cocktails (Figure 3d), the expression of white adipocyte markers was not significantly influenced by Grem2 (Figure 3e). In further, Grem2 inhibited the basal, uncoupled, and maximal oxygen consumption rates (OCRs) of induced beige adipocytes (Figure 3f). In consistence, Grem2 treatment also inhibited Ucp1 mRNA expression in the differentiated beige adipocytes derived from human visceral fat SVFs (Figure 3g).

**Figure 2.** Plasma Grem2 levels change in response to various external stimuli. a. Relative mRNA levels of Grem2 in various tissues of mice (n = 3–6 per tissue). b. Plasma Grem2 levels in mice with surgical removal of bilateral eWATs (n = 8). c. 24 h fasting challenge induces plasma Grem2 reduction and refeeding promotes plasma Grem2 rebound in mice (n = 3). d. Plasma Grem2 levels in ob/ob mice (n = 9–10). e. Relative mRNA levels of Grem2 in eWAT of ob/ob mice (n = 9–10). f–g. The isolated visceral fat was incubated in conditioned media for 6 h that were sampled at 1 h, 3 h and 6 h, respectively. The Grem2 protein was detected by western blotting (f) and ELISA (g) methods (n = 7 for ob/ob and WT mice). h. Plasma Grem2 levels after short-term (24 h) and long-term (10 days) cold exposure (n = 6 for controls, 6 for short-term and 7 for long-term, respectively). i. Grem2 protein expression in eWAT, iWAT and BAT after 10-day cold exposure. j. Relative mRNA expression of Grem2 in SVFs and mature adipocytes (MAs) derived from eWAT from NCD-fed mice (left) and from HFD-fed mice (right) (n = 3). k. Relative mRNA expression of Grem2 after beige adipocyte induction of eWAT-derived SVFs (n = 4). l. Grem2 protein levels in conditioned medium after beige adipocyte induction of eWAT-derived SVFs (n = 3). For c and h, one-way ANOVA was used for the multiple group comparisons. For b, d, e, g and j, two-tailed Student’s t-test was used for two group comparisons. Data are shown as mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.005; **** p < 0.001.
These results suggest that Grem2 may show a moderately inhibitory effect on the browning ability of visceral fat-derived preadipocytes in vitro. Further supporting evidence was collected in Grem2 transgenic (Grem2\(^{Tg}\)) mice (Supplementary Fig. 5a). Grem2 was overexpressed in eWAT (Figure 4a,b) and other tissues (Supplementary Fig. 5b–d). Compared with WT mice, Grem2\(^{Tg}\) mice showed no significant changes in glucose tolerance or food intake (Supplementary Fig. 5e,f). Despite the detection of unchanged energy expenditure, including O\(_2\) consumption, CO\(_2\) production, or physical activity under normal condition
Figure 4. Attenuated browning abilities of visceral fat in Grem2 transgenic (termed Grem2Tg) mice. a–b. Relative mRNA (a) and protein (b) levels of Grem2 in eWAT of Grem2Tg and WT mice, respectively (n = 8–10). c. Relative mRNA levels of thermogenic genes in the eWAT of both groups under cold exposure for 10 days (n = 3–5). d. Representative western blotting (left) and the quantification (right) of Ucp1 levels in eWAT of both groups for 10-day coldness. HSP90 were used as internal controls. e. Representative H&E and immunohistochemical (IHC) staining of Ucp1 in the eWAT treated as (d). Scale bar, 100 μm. f–h. Relative mRNA levels of Grem2 and thermogenic genes (n = 5) (f), the protein levels of Grem2 and Ucp1 (left), and their quantification (right) (g), and OCRs measurement (n = 4) (h) in fully differentiated eWAT-derived beige adipocytes of both groups ex vivo. For a, c, d, f–h, two-tailed Student’s t-test was used for two group comparisons. Data are shown as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.005.
(Supplementary Fig. 5g–i), the expression of browning-related genes was repressed in eWAT of Grem2−/− mice after 10 days of cold stimulation (6 °C) (Figure 4c). No significant change of adipogenesis and chronic inflammation genes was detected (Supplementary Fig. 5j,k). Under cold stress, Ucp1 protein expression was mildly attenuated by Grem2 overexpression (Figure 4d), and Grem2−/− mice exhibited weaker Ucp1 staining and larger adipocytes compared with those of WT mice (Figure 4e). By inducing eWAT-derived SVFs of Grem2−/− and WT mice into mature beige adipocytes, we also observed a moderately repressed Ucp1 expression (Figure 4f,g) and decreased uncoupled OCR levels (Figure 4h) in Grem2-overexpressed beige adipocytes.

Grem2 ablation mildly enhances the browning capacity of visceral fat
We further generated adipose Grem2 knockout mice by crossing Grem2floxflox mice with aP2-cre mice (Grem2floxflox;aP2-cre, termed AGKO mice; Supplementary Fig. 6a), as aP2-cre was reported to delete target genes mainly in adipocytes and a proportion of preadipocytes.36 As expected, AGKO mice showed Grem2 deficiency in eWAT (Figure 5a) and other adipose tissues (Supplementary Fig. 6b,c). In growth curves under NCD, AGKO mice displayed mildly decreased body weights (Figure 5b) as well as reduced visceral fat mass (Figure 5c and Supplementary Fig. 6d), while no significant change in blood glucose, food intake, and energy expenditure was observed under nomal condition (Supplementary Fig. 6e–i). No obvious change of adipogenesis and chronic inflammation genes was observed (Supplementary Fig. 6j,k). Of note, under cold stress, enhanced browning features such as increased Ucp1 mRNA and protein expression and smaller adipocytes with Ucp1 positive staining were observed in eWAT of AGKO mice (Figure 5d–f). These data indicate that ablation of Grem2 mildly promotes the browning capacity of visceral fat and is associated with reduced visceral fat content.

Grem2 binds to BMP4/7 and inhibits the browning capacity of visceral preadipocytes partially via the BMP4/7-BMPR2-SMADs signaling pathway
Consistent with previous findings that demonstrated the promotional roles of BMP4 and BMP7 in the browning progress of sWAT,5,8 in this study, we found that both BMP4 and BMP7 also enhanced the browning capacity of eWAT-derived SVFs in a concentration-dependent manner in vitro (Supplementary Fig. 7a,b). Then, recombinant Grem2 protein was combined with BMP4 and BMP7 treatment, respectively, to treat preadipocytes for 3 days before induction. Interestingly, we found that Grem2 significantly attenuated the promotional effects of BMP4 and BMP7 on the expression of Ucp1 and other browning-related genes (Figure 6a–d). Using Oil Red O staining, we also revealed that both BMP4 and BMP7 promoted lipid droplet formation in mature beige adipocytes, while the preadipocytes treated with Grem2 alone or in combination with BMP4/7 showed fewer lipid droplets than the corresponding control groups (Supplementary Fig. 7c,d). Next, we revealed that Grem2 could interact to both BMP4 and BMP7 proteins (Supplementary Fig. 7e,f). The binding of BMP dimers to their receptor complex activates the type II receptor (BMPRII in adipose tissue) to phosphorylate the type I receptor (BMPRI), which in turn activates the regulatory canonical SMAD pathway or p38 in the non-canonical pathway.39,40 To evaluate the potential involvement of the BMP receptor complex of preadipocytes in the process of Grem2 antagonizing BMP4/7, we generated preadipocyte Bmpr2 knockout mice by crossing Bmpr2f/f mice7 with Pdgfra-cre mice16 (Bmpr2−/−;Pdgfra-cre, termed pBRKO). Following the induction of beige adipocyte cocktails ex vivo, the browning capacity of visceral preadipocytes was largely attenuated in pBRKO mice compared with control mice (Figure 6e–h). Furthermore, Grem2 could not inhibit the mRNA and protein expression of Ucp1 in differentiated beige adipocytes derived from Bmpr2-deficient preadipocytes; and more importantly, the antagonizing effects of Grem2 on BMP4/7’s browning ability was largely attenuated under the Bmpr2-deficient condition. Finally, we found that both BMP4 and BMP7 enhanced the phosphorylation of SMAD1/5/8 in visceral preadipocytes, which could be repressed by additional Grem2 treatment; however, the p38MAPK signaling pathway was not inhibited by Grem2 treatment (Figure 6i,j). In addition, Grem2 could not antagonize BMP4/7’s effects on canonical Wnt signaling (Supplementary Fig. 8a,b) or the expression of Wnt downstream target genes (Supplementary Fig. 8c,d). Taken together, these results suggest that the inhibitory role of Grem2 in the browning process of visceral fat could be, in part at least, attributed to its inhibition of the BMP4/7-BMPR2-SMAD signaling pathway in visceral preadipocytes.

Discussion
In this study, we performed translational research to identify a circulating protein associated with visceral adiposity. Using an ELISA method, we measured circulating GREM2 levels in three independent cohorts, and reported that GREM2 was elevated in obese individuals and was more strongly associated with central obesity and visceral fat content than general obesity. Interestingly, visceral fat secreted more Grem2 into extracellular fluids in adiposity status, and circulating Grem2 levels were associated with the changes of visceral fat content and fluctuated in response to external calorie supplies. Moreover, using transgenic mouse models, we revealed that Grem2 overexpression reduced the
The concept of central obesity was first proposed by Vague et al. in the 1940s. Central obesity is considered a stronger independent risk factor for cardiometabolic disorders than general obesity. The NHANES III cohort study disclosed that subjects with normal-weight central obesity had the worst long-term survival. In contrast to the routine index of central obesity (such as WC and WHR), the exact quantification of visceral fat mass and content with whole-visceral cavity CT or Magnetic Resonance Imaging (MRI) scanning is scarce at present clinical practices, which also impedes the identification of visceral adiposity-related serum biomarkers. This study demonstrated the association between circulating GREM2 levels and visceral fat mass with a
Figure 6. Grem2 inhibits the browning capacity of visceral preadipocytes partially via BMP4/7-BMPR2 signaling pathway. a-d. Relative mRNA levels of thermogenic genes (n = 4–5) (a and b) and the protein levels of Ucp1 (c and d) in induced beige adipocytes from visceral preadipocytes pre-treated with Grem2 in addition to BMP4 (a and c) or BMP7 (b and d), respectively, for 3 days before induction. e-h. The mRNA expression of thermogenic genes (n = 4–6) (e and g) and the protein level of Ucp1 (f and h) in induced beige adipocytes from Bmpr2fl/flxp/flxp-pdgfrα-cre (termed pBRKO) and Bmpr2fl/flxp/flxp-derived visceral preadipocytes pre-treated with Grem2 in addition to BMP4 (e and f) or BMP7 (g and h), respectively, for 3 days before induction. i-j. The protein levels of phospho-SMAD1/5/8 and p38MAPK proteins in visceral preadipocytes treated with Grem2 in addition to BMP4 (i) and BMP7 (j), respectively, for one hour. For a-b, e and g, one-way ANOVA was used for the multiple group comparisons. Data are shown as mean ± SEM. *, p < 0.05; **, p < 0.01; ***,***, p < 0.001.
also promote the differentiation and browning capacity of visceral preadipocytes. Notably, Grem2 could bind to BMP4/7 and attenuated the promoting effects of BMP4/7 on cell fate determination to browning adipocyte differentiation. Other studies have demonstrated that downstream SMAD1/5/8 signaling has a role in lineage determination, whereas p38/MAPK signaling only shows minor effects on commitment efficiency.

It was assumed that the various local-concentration ratios of BMP antagonists (such as GREM1/2) to BMPs (like BMP4/7) might contribute to biased activation of downstream pathway and further the intrinsic browning capacity of preadipocytes in different fat depots, which also needs further investigations.

A mature BMPs-BMPR signaling complex requires one ligand dimer firstly to bind two type II receptors and then recruit two type I receptors. Brown fat pacity due to ablation of Bmpr1a induces a robust compensatory browning change in white fat, whereas the ablation of Bmpr2 in preadipocytes (Pdgfrα) has not yet been reported. This study found that pBRKO mice showed a similar growth and body weight phenotype to our previously reported adipose tissue Bmpr2 knockout mice but had an earlier defect in WAT development. The preadipocytes derived from visceral fat depots of pBRKO mice exhibited a weaker differentiation capacity of beige adipocytes than those of control mice. In the Bmpr2-deficient context, the antagonistic effects of Grem2 on BMP4/7 in the adipogenesis of beige adipocytes were largely attenuated, suggesting an important role of GREM2-BMP4/7-BMPR2-SMAD signaling in the modulation of the inert browning capacity of visceral fat. However, whether other BMPs and BMP receptors are also involved in the process remains unclear, and the way Grem2-BMP4/7-BMPR2 signaling axis specifically functions on the visceral fat but not on other fat depots also warrants further investigation.

It should be noted that we did not detect any significant energy expenditure changes either in Grem2-transgenic or Grem2 knockout mice. One possible explanation is that the contribution of visceral fat browning in whole-body energy expenditure might be too subtle to be detected in mice by the presently available instruments, as we indeed revealed changes in mitochondrial respiration in Grem2-transgenic beige adipocytes ex vivo. Given that a one-unit increase in visceral fat mass was associated with a 2.7-fold higher risk of type 2 diabetes, selectively targeting visceral fat in clinical practice is promising and would more effectively reduce the complications related to visceral adiposity. In combating visceral obesity, a probability remains that improving the browning ability of visceral fat, such as by functional blockade of GREM2, may lead to long-term metabolic benefits. Of note, several physiological and biological questions regarding the roles of blood Grem2 on metabolic traits remain to be answered. For instance, which tissues are the main source and target of blood Grem2, and whether Grem2...
could function in an endocrine way to regulate fat mass. Finding answers to these important questions will provide new insights not only into visceral adiposity but also into the whole-body signaling network that maintains metabolic homeostasis during obesity.

Conclusion
This study reports that GREM2 appears to be a circulating protein factor as an independent variable correlated to visceral obesity. In addition, we suggest a potential molecular mechanism underlying Grem2 inhibiting the lowering capacity of visceral preadipocytes. These findings highlight the possibility of targeting GREM2 and its downstream pathways for obesity stratification and clinical intervention of human visceral adiposity.

Contributors
Conceptualization, J.Q.W.; Methodology, J.Q.W., W.Liu, D.L., and L.W.; Experiment, W.Liu, D.L., M.L.Y., R.X.L., L.W., Y.X., and Z.Y.Z.; Material, S.W.Q, and Q.Q.T.; Investigation, W.Li, S.Q.Z., A.B.G., N.C., Q.Y.M., R.Z., and S.J.W.; Writing – Original Draft, W.Liu and L.W.; Writing – Review & Editing, J.Q.W. and R.X.L.; Funding Acquisition, J.Q.W., G.N., W.Q.W., R.X.L.; Y. Hong.C., and W.Liu; Human Resources, J.Q.W., G.N., W.Q.W., Y.X., J.H., W.Q.G, Y.F.Z, J.S., Y.Fei.C., Y. Hong.C., and Y.F.B., W.Liu, D.L., M.L.Y., L.W. and Y.X; verified the underlying data. D.L. and R.X.L. provided critical feedback in the revision of the manuscript. All authors read and approved the final manuscript.

Data sharing statement
The expression profile of visceral WAT in Lgr4-deficient mice has been deposited in GEO (GSE195570). Summary data are available in the paper and in supplementary materials. Raw data that support the findings of this study are available from the corresponding author upon request.

Declaration of interests
The authors have declared that no conflict of interest exists.

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Supplementary materials
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References
1 Collaboration NCDRF. Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 169 population-based measurement studies with 19.2 million participants. Lancet. 2016;387(10036):1377–1396.
2 Cypess AM. Reassessing Human Adipose Tissue. N Engl J Med. 2012;366(8):68–779.
3 Diéguez-Díez D, Tomás-Moya I, Gómez-Aguirre D, et al. The cell biological and functional complexity of thermogenic fat. Nat Rev Mol Cell Biol. 2022;23(6):391–409.
4 El-Chafy H, Duan Y, Qian SW, et al. BMP4-mediated brown fat-like adipose tissue plasticity. J Am Coll Cardiol. 2019;74(10):1212–1263.
5 Fan X, Xiao F, Wang CD, et al. Gremlin 2 inhibits adipocyte differentiation and its inhibitory effect on adipogenesis. J Biol Chem. 2013;288(52):38678–38692.
6 Guoqing Bao from Sydney University for his help in visceral fat 3D reconstruction. Funding: This work was supported by grants from the National Natural Science Foundation of China (91957124, 81730023, 92157204, 91857205, 82088102, 81822009, 81930021, 81870585, 81870604, 81800747, 81870506, and 82000814), the National Key Research and Development Program of China (2018YFC1313800), Shanghai Municipal Education Commission-Gaofeng Clinical Medicine Grant Support (20161306 and 20171903), Shanghai Yangfan Program (19YF1428500), and Program of Shanghai Academic/Technology Research Leader (20XD1403200).

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2 Cypess AM. Reassessing Human Adipose Tissue. N Engl J Med. 2012;366(8):68–779.
3 Diéguez-Díez D, Tomás-Moya I, Gómez-Aguirre D, et al. The cell biological and functional complexity of thermogenic fat. Nat Rev Mol Cell Biol. 2022;23(6):391–409.
4 El-Chafy H, Duan Y, Qian SW, et al. BMP4-mediated brown fat-like adipose tissue plasticity. J Am Coll Cardiol. 2019;74(10):1212–1263.
5 Fan X, Xiao F, Wang CD, et al. Gremlin 2 inhibits adipocyte differentiation and its inhibitory effect on adipogenesis. J Biol Chem. 2013;288(52):38678–38692.
6 Guoqing Bao from Sydney University for his help in visceral fat 3D reconstruction. Funding: This work was supported by grants from the National Natural Science Foundation of China (91957124, 81730023, 92157204, 91857205, 82088102, 81822009, 81930021, 81870585, 81870604, 81800747, 81870506, and 82000814), the National Key Research and Development Program of China (2018YFC1313800), Shanghai Municipal Education Commission-Gaofeng Clinical Medicine Grant Support (20161306 and 20171903), Shanghai Yangfan Program (19YF1428500), and Program of Shanghai Academic/Technology Research Leader (20XD1403200).

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26 Zou Y, Ning T, Shi J, et al. Association of a gain-of-function variant in LGR4 with central obesity. *Diabetes*. 2017;66(4):935–947.

27 Qian SW, Wu MY, Wang YN, et al. BMP4 facilitates beige fat biogenesis in vivo and in vitro. *Acta Diabetol*. 2019;56(1):24–32.

28 Chen M, Lu P, Ma Q, et al. Association of a gain-of-function variant in LGR4 with central obesity. *Obesity*. 2017;25(6):1245–1250.

29 Parikh RM, Joshi SR, Pandia K. Index of central obesity is better than waist circumference in defining metabolic syndrome. *Obes Res*. 2007;15(4):920–926.

30 Amato MC, Giordano C, Calia M, et al. Visceral adiposity index: a reliable indicator of visceral fat function associated with cardiometabolic risk. *Diabetes Care*. 2010;33(4):920–926.

31 Jensen MD, Ryan DH, Apovian CM, et al. AHA/ACC/TOS guideline for the management of overweight and obesity in adults: a report of the American college of cardiology/American heart association task force on practice guidelines and the obesity society. *J Am Coll Cardiol*. 2014;64(12 Pt B):1085–1129.

32 Pan XF, Wang L, Pan A. Epilepsiology and determinants of obesity in China. *Lancet Diabetes Endocrinol*. 2021;9(6):377–392.

33 Karlsson T, Rask-Andersen M, Pan G, et al. Contribution of genetics to visceral adiposity and its relation to cardiovascular and metabolic disease. *Nat Med*. 2019;25(9):1390–1395.

34 Lotta LA, Wittmanns LB, Zuber V, et al. Association of genetic variants related to glucose metabolism vs abdominal fat distribution with type 2 diabetes, coronary disease, and cardiovascular risk factors. *JAMA*. 2018;320(24):2533–2543.

35 Ding H, Zheng S, Garcia-Ruiz D, et al. Fasting induces a subcutaneous-to-visceral fat switch mediated by microRNA-149-3p and suppression of PDK4. *Nat Commun*. 2016;7:11533.

36 Knueger KC, Costa MJ, Du H, Feldman BJ. Characterization of Cc recombinase activity for in vivo targeting of adipocyte precursor cells. *Stem Cell Rep*. 2014;3(6):1147–1158.

37 Schulz Tj, Huang TL, Tran TT, et al. Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. *Proc Natl Acad Sci U S A*. 2011;108(1):613–618.

38 Hoffmann JM, Grunberg JR, Church C, et al. BMP4 gene therapy in mature mice reduces BAT activation but protects from obesity by increasing subcutaneous adipose tissue. *Cell Rep*. 2017;20(5):1019–1029.

39 Blazquez-Medela AM, Jumabai M, Bostrom KI. Beyond the bone: Bone morphogenetic protein signaling in adipose tissue. *Obes Rev*. 2010;11(5):548–568.

40 Schmieder B, Hill CS. TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol*. 2005;6(12):987–992.

41 Vague J. A determinant factor of the forms of obesity. *Obes Res*. 1996;4(2):201–203.

42 Sahakyan KR, Somers VK, Rodriguez-Escudero JP, et al. Normal-weight central obesity: implications for total and cardiovascular mortality. *Ann Intern Med*. 2015;163(9):627–635.

43 Hedjazifar S, Khatib Shahidi R, Hammarstedt A, et al. The Novel adipokine gremlin 1 antagonizes insulin action and is increased in type 2 diabetes and NAFLD/NASH. *Diabetes*. 2020;69(3):331–341.

44 Elsen M, Raschke S, Tennagels N, et al. BMP4 and BMP7 induce the white-to-brown transition of primary human adipose stem cells. *Am J Physiol Cell Physiol*. 2014;306(3):C417–C426.

45 Huang H, Song TJ, Li X, et al. BMP signaling pathway is required for commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. *JAMA*. 2011;306(13):12670–12675.

46 Schulz Tj, Huang P, Huang TL, et al. Brown-fat paucity due to impaired BMP signalling induces compensatory browning of white fat. *Nature*. 2014;510(748):579–583.

47 Qian S, Pan J, Su Y, et al. BMP2 promotes fatty acid oxidation and protects white adipocytes from cell death in mice. *Commun Biol*. 2020;3(1):200.

48 Karlsson T, Rask-Andersen M, Pan G, et al. Contribution of genetics to visceral adiposity and its relation to cardiovascular and metabolic disease. *Nat Med*. 2019;25(10):1390–1395.

49 Rodriguez A, Catalan V, Gomar-Benitez J, Fruhbeck G. Visceral and subcutaneous adiposity: are both potential therapeutic targets for tackling the metabolic syndrome? *Curr Pharm Des*. 2007;13(21):2165–2175.

50 Smith SR, Zachwieja JJ. Visceral adipose tissue: a critical review of intervention strategies. *Int J Obes Relat Metab Disord*. 1999;23(4):324–335.