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FGF9 inhibits browning program of white adipocytes and associates with human obesity

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Abstract

Browning of white adipose tissue has been proven to be a potential target to fight against obesity and its metabolic commodities, making the exploration of molecules involved in browning process important. Among those browning agents reported recently, FGF21 play as a quite promising candidate for treating obesity for its obvious enhancement of thermogenic capacity in adipocyte and significant improvement of metabolic disorders in both mice and human. However, whether other members of fibroblast growth factor (FGF) family play roles in adipose thermogenesis and obese development is still an open question. Here, we examined the mRNA expression of all FGF family members in three adipose tissues of male C57BL/6 mice and found that FGF9 is highly expressed in adipose tissue and decreased under cold stress. Furthermore, FGF9 treatment inhibited thermogenic genes in the process of beige adipocytes differentiation from stromal vascular fraction (SVF) in a dose-dependent manner. Similar results were obtained with FGF9 overexpression. Consistently, knockdown of FGF9 in SVF cells by using lentiviral shRNA increased thermogenic genes in differentiated beige adipocytes. RNA sequencing analysis revealed a significant increment of hypoxia-inducible factor (HIF) pathway in the early stage of beige adipocytes differentiation under FGF9 treatment, which was validated by real-time PCR. FGF9 expression was increased in subcutaneous WAT of obese human and mice. This study shows that adipose-derived FGF9 play as an inhibitory role in the browning of white adipocytes. Activation of hypoxia signaling at early stage of adipose browning process may contribute to this anti-thermogenic effect of FGF9.

Key Words

- ▶ FGF9
- ▶ browning
- ▶ HIF1 α
- ▶ obesity

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Introduction

Obesity is becoming one of the major concerns of modern society for its increasingly economic burden and various associated metabolic disorders. Although the medical research has been achieved a great development in recent decades, the most recent study still revealed that the average BMI is rapidly increased worldwide in the past 40 years

(Collaboration NCDRF 2016). Exploration of the potential targets for obesity prevention is still largely warranted.

Obesity results from excess energy intake over expenditure, which causes more energy storage in white adipose tissues (WATs). Brown adipocytes can dissipate energy into heat in response to certain stimuli such as cold

exposure, beta3-AR agonists by uncoupling respiratory electron transport chain, giving a promising therapeutical target for obesity and its commodities (Bartelt & Heeren 2014). Two distinct kinds of adipose tissues have been identified as the major site for this thermogenic process (Bartelt & Heeren 2014), brown adipose tissues (BAT) and interspersed brown-like adipocytes (also called 'beige' or 'brite' cells) within WATs. Compared to classical BAT that located in interscapular region, the beige adipocytes show greater therapeutic potential for its 'inducible-brown' property, which also called browning of WAT. Brown fat has been recently discussed extensively for the validation of its existence in human and great potential for treating obesity (Virtanen *et al.* 2009).

Numerous factors that are involved in this browning process have been identified recently (Bartelt & Heeren 2014). Among these various factors, several adipokines, including BMP4, BMP7 and FGF21, have been proven as critical regulators (Tseng *et al.* 2008, Fisher & Maratos-Flier 2016), suggesting the importance of adipocytes itself in regulating this thermogenic process. Of note, according to recent studies, FGF21, a secretory protein belonging to fibroblast growth factor (FGF) family, exhibited a great potential to promote thermogenic capability in both brown and beige adipocytes (Chartoumpekis *et al.* 2011, Hondares *et al.* 2011, Fisher *et al.* 2012, Fisher & Maratos-Flier 2016), indicating a crucial involvement of this signaling protein in adipose thermogenesis. Even though several experiments have challenged its endogenous browning effect (Keipert *et al.* 2017), those previous findings that exogenous treatment of FGF21 could promote adipose thermogenesis significantly still make this molecule a pharmacological candidate for regulating browning of adipocyte. In addition, treating with several FGF21 analogs showed a great metabolic benefits in both animals and human (Gaich *et al.* 2013, Talukdar *et al.* 2016), further suggesting the pharmacological potential of FGF21 to improve energy metabolism. Meanwhile, some other members of FGF family, including FGF1, FGF19, FGF23 and so forth, exhibited metabolic involvement as well (Shimada *et al.* 2004, Song *et al.* 2009, Kir *et al.* 2011, Suh *et al.* 2014, Degirolamo *et al.* 2016). Especially FGF1, which has been proven to be able to remodel WAT (Jonker *et al.* 2012, Sun & Scherer 2012) and act as a potential candidate for treating diabetes for its potent insulin-sensitizing effect (Suh *et al.* 2014, Gasser *et al.* 2017). These findings implied that FGF family may play important roles in energy metabolism, most of which have not yet been clearly determined.

In this study, we focused on the metabolic role of FGFs in adipose tissues and aim to identify new regulators of

the adipose browning effect among other members of FGF family. We screened the expression of all 22 FGF family members in brown adipose, inguinal and epididymal WAT respectively, finding several FGFs that highly expressed in all of three types of adipose tissues. Among the selected candidates, FGF9 treatment significantly inhibited the expression of thermogenic genes and adipogenic genes, indicating the inhibitory effect of FGF9 on the browning of WAT. Further experiments confirmed this suppressive effect of FGF9 on adipose thermogenesis. Moreover, we also found that the FGF9 expression increased in obese human and mice, implying that FGF9 may play a potential role in obesity by inhibiting thermogenic capacity of adipose tissue. Our findings identify FGF9 as a novel regulator on browning and provided a potential target for treating obesity.

Materials and methods

Animals

Male mice with C57BL/6 background were obtained from Shanghai Slake experimental animal corporation and housed in SPF environment at 22±2°C with 12h/12h light–darkness cycles, grouped with 3–4 mice per cage. Experimental mice were scarified when 6–8 weeks old for tissue collection and adipose SVF isolation. In some experiments, mice aged 8 weeks were fed with 60kcal% HFD (Research Diet) for 4 months and then scarified for experiments.

SVF and mature adipocytes isolation

Adipose tissues were isolated and minced before digestion with 1mg/mL, type II collagenase (Sigma) in DMEM (Invitrogen) supplemented with 1% bovine serum albumin for 35min at 37°C. After digestion, the cell suspensions were allowed to stand on ice for 10min to terminate the digestion and then centrifuged at 1153g for 10min at 4°C. The floating mature adipocytes fraction were harvested for further detection, the precipitate was washed with DMEM medium, and then filtered by using 40µm strainer (BD Biosciences) before planted into 6 or 10cm cell dish.

SVF culture and incubation

The planted SVF were cultured with growth medium composed by DMEM medium, 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen) to

reach confluence, and then digested by using trypsin to be replanted into cell culture plate on the basis of experimental design. The replanted cells were cultured with growth medium until re-reach confluence, and incubated with brown adipocytes differentiation cocktail, including 6 µg/mL insulin, 0.5 mM isobutylmethylxanthine (Sigma), 1 µM dexamethasone (Sigma), 5 nM T3 (Sigma) and 5 µM troglitazone (Sigma) in the first 48 h, and insulin, T3 and troglitazone in following 6 days. After full differentiation, the cells were collected by using TRIzol reagent for RNA extraction, and RIPA for protein extraction respectively. Recombinant human FGF9 (R&D) was absent or present in accordance to experimental design after cells were replanted. More details refer to previous study (Wang *et al.* 2013).

Adenoviral and lentiviral infection

For adenoviral and lentiviral transduction, SVFs isolated from inguinal adipose tissue were transfected with either adenovirus vectors overexpression homo-Fgf9 using 1 mg/mL linear polyethylenimine transfection reagent or lentiviral contain shRNA targeted Fgf9 using 5 µg/mL polybrene. Viral supernatants were collected and replaced with fresh media 24 h after infection. The sequence of shRNA used in this study was provided in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article).

Oxygen consumption rate measurements

SVFs isolated from iWAT were plated in a XF24-well microplate (Seahorse Bioscience) and incubated into brown adipocytes for 8 days, followed by oxygen consumption rate (OCR) measurement at 37°C by using XF24 analyzer (Seahorse Bioscience) in accordance with the manufacturer's instructions. 1 µM oligomycin, 2 µM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone and 1 µM rotenone/antimycin were delivered to detect the uncoupled respiration, maximal respiration and nonmitochondrial respiration, respectively. The final OCR in each well was corrected by its protein concentration, which was detected after finish OCR measurement.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from cells or tissues using TRIzol reagent in accordance with the manufacturer's instructions. 1 µg RNA was transcribed to complementary DNA with Reverse Transcription System (Promega).

Real-time PCR was carried out by the LC480 system (Roche) using SYBER Green Supermix (Takara). Primers used in this study were provided in Supplementary Table 1.

Protein preparation and Western blot

Proteins from cells were prepared using RIPA buffer. The concentration of all protein samples was determined before protein denaturation with 99°C for 10 min. The denatured proteins were subjected to immunoblot assay with UCP1 antibody (diagnostic alpha, UCP11-A) and PGC1α (Millipore, AB3242), HSP90 was used as the internal controls. The bands were visualized by using Odyssey infrared imaging system (LI-COR) according to the manufacturer's guide.

Oil Red O staining

Fully differentiated SVFs were washed twice with PBS before fixed with 4% paraformaldehyde for 20 min, and then incubated with Oil Red O (Sigma) for 30 min at 37°C.

RNA sequencing analysis

The RNA sequencing was performed by using HiSeq 2500 system. We analyzed the sequencing data by employing several R packages, including 'DeSeq2' for the comparison of differential genes, 'ProfilerCluster' for Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis, and 'ggplot2' for better visualization of data. The threshold for picking differential genes is adjusted *P* value <0.01 and Log2 (fold change) >1; the threshold for determining the alteration of pathway is *P* value <0.05.

Patients

Twenty-one obese patients (age: 18–38, Sex: 9 males and 12 females, BMI ≥35 kg/m²) who were undergone sleeve gastrectomy and 11 normal-weight patients (age 18–30, sex: four males and seven females, BMI ≤24 kg/m²) with non-malignant diseases were recruited in this study, we collected their visceral and subcutaneous adipose tissues in the process of surgery, storing in liquid nitrogen for further study. This study was approved by the Institutional Review Board of the Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, and was in accordance with the principle of the Helsinki Declaration II. Written informed consent was obtained from each participant.

Statistical analysis

Data are presented as the means $\pm$ S.E.M. Significant difference was indicated by *P* value <0.05 in two-tailed Student's *t*-test analysis.

Results

FGF9 is widely expressed in adipose tissues

To screen the potential function of FGFs in adipose tissues, we first examined the gene expression of all FGF family members in iWAT, eWAT and BAT. Among them, FGF9, FGF10 and FGF14 showed overall high expression in three adipose tissues, while FGF21, previously reported to promote browning, showed moderate expression (Fig. 1A, B and C). We next selected one member of each secreted-FGFs subfamilies that highly expresses in both WAT and BAT to further test their effects on browning. Fgf4 and Fgf8 subfamily were excluded for their comparatively low expression, Fgf11 subfamily were excluded for its intracellular property, and FGF1 were excluded for its strong effect on adipose remodeling, which has been reported recently (Jonker *et al.* 2012). Finally, three members, FGF9, FGF10 and FGF23 were selected. Another BAT-enriched protein FGF14, belonging to Fgf11 subfamily, was excluded for its intercellular property, which limits its pharmacological effects. We used recombinant human FGF9, FGF10 and FGF23 to treat isolated SVF and incubated them with brown-adipocyte-induction cocktail. Interestingly, FGF9 treatment significantly inhibited brown adipocyte marker Ucp1 expression (Fig. 1D), suggesting the potential inhibitory role of FGF9 on browning. We next focused on FGF9 to conduct further experiments. We examined the expression pattern of FGF9 in multiple tissues of male C57BL/6 mice and found that FGF9 was widely expressed in various metabolic tissues and highly expressed in eWAT and kidney (Fig. 1E). These results demonstrated that FGF9 may play potentially important effects on adipocytes function.

FGF9 inhibits the browning of white adipocytes in a dose-dependent manner

We next used different experiments to examine the effects of FGF9 on the browning process of white adipocytes. We observed that FGF9 expression was significantly decreased in inguinal WAT of mice in response to cold-induced thermogenesis and epididymal WAT of mice in response to β 3-adrenoceptor agonist's stimulus (Fig. 2A and B).

Figure 1

FGF9 was highly expressed in adipose tissues. (A, B and C) mRNA levels of FGF family members in brown adipose tissue ($n = 3$) (A), inguinal white adipose tissue ($n = 5$) (B) and epididymal white adipose tissue ($n = 6$) (C). (D) mRNA levels of Ucp1 expression in fully differentiated SVF (day 8 after incubation) isolated from inguinal white adipose tissue of mice ($n = 4$) under browning incubation in response to treatment of FGF9, FGF10, and FGF23. (E) mRNA level of FGF9 in multiple tissues of mice ($n = 3-6$).

These results supported the inhibitory role of FGF9 in browning. Based on the previous reports that adipocytes with browning capacity originated from SVF of adipose tissues, we further isolated stromal vascular fraction (SVF) cell from subcutaneous WAT of mice and incubated them toward brown adipocytes with or without recombinant human FGF9. Significantly, FGF9 treatment inhibited beige adipocytes differentiation, as reflected by a decrease in Red O oil staining (Fig. 2C) and a dose-dependent inhibition in gene expression involved in thermogenesis including Ucp1, Pgc1 α , Cidea, and Prdm16, meanwhile, adipogenesis biomarkers, including C/ebp β , C/ebp α , ppary and Fabp4, were suppressed in both beige and white differentiation (Fig. 2D, E, F, G, H, I, J and K and

Figure 2

FGF9 inhibits adipose thermogenesis in a dose-dependent manner. (A) mRNA level of FGF9 expressed in iWAT in mice ($n = 3$) under cold condition (4°C). (B) mRNA level of FGF9 expressed in eWAT in mice ($n = 10$) in response to CL316243 (1.5 mg/kg, 10 days injection). (C) Oil Red staining of full-differentiated SVF (8th day after incubation) toward brown in the absence and presence of FGF9 (100 ng/mL). (D, E, F, G, H, I, J and K) mRNA of thermogenic biomarkers (D, E, F and G) and adipogenic biomarkers (H, I, J and K) in full-differentiated SVF isolated from mice iWAT in response to gradient concentration of FGF9. (L and M) Protein level of UCP1 and oxygen consumption rate (OCR) of full-differentiated SVF in response to FGF9 treatment (100 ng/mL). (N) mRNA level of browning genes in full-differentiated human SVF under different dose of FGF9. (O) mRNA level of thermogenic and adipogenic genes profile under condition of FGF9 overexpression. (P) Protein level of UCP1 and PGC1 α in response to FGF9 overexpression. (Q) Oxygen consumption rate in response to FGF9 overexpression.

Supplementary Fig. 1). A substantial reduction of UCP1 protein, and OCRs in both basal and uncoupled status have been observed in full-differentiated SVF under beige incubation in response to FGF9 treatment (Fig. 2L and M). Moreover, we also examined the inhibitory effect of FGF9 in human SVF, in which we observed a significant reduced Ucp1 expression under high concentration of FGF9. To further validate this inhibitory effect of FGF9 on the browning process of SVF, we constructed adenovirus overexpressing FGF9 and transfected it into undifferentiated SVF followed by induction into brown adipocytes. Consistently, we detected significantly decreased gene expression in the above thermogenic genes under FGF9 overexpression (Fig. 2N and O). Basal and uncoupled OCR was also reduced by FGF9 overexpression (Fig. 2P). These results demonstrated that FGF9 inhibited the browning process of white adipocytes.

FGF9 suppression promotes browning of white adipocytes

After determination of the anti-thermogenic effect of exogenous FGF9 or overexpression of FGF9, we next explored whether FGF9 could participate in inhibiting the browning process endogenously. Using two FGF9 lentiviral

short hairpin RNA (shRNA), we efficiently suppressed FGF9 expression in different time points during browning differentiation. (Fig. 3A) The fully differentiated adipocytes with reduced FGF9 expression showed increased Red O oil staining (Fig. 3B) and expression of Ucp1, Pgc1 α , Cidea and PRDM16. We did not observe a significant alteration in adipogenic gene expression (Fig. 3C). The Ucp1 protein was also significantly increased upon FGF9 knockdown (Fig. 3D). Concomitantly, OCR was elevated in response to FGF9 reduction (Fig. 3E and F). These results demonstrated that FGF9 suppression promoted the browning of adipose tissues.

FGF9 suppresses thermogenic genes from the early stage of adipocytes differentiation, following a provocation of HIF1 α pathway

According to previous studies, the maturation process of SVF includes commitment and differentiation phase (Tang & Lane 2012), which both contributes to the browning process of white adipocytes (Bartelt & Heeren 2014). Thus, we firstly examine the dynamic changes of thermogenic genes in both of commitment and differentiation stage during SVF differentiation toward brown adipocytes under FGF9 treatment. As we observed, the suppressive

Figure 3

FGF9 suppression promotes browning of white adipocytes. (A) mRNA expression of Fgf9 in different time points during browning differentiation under two Fgf9-shRNA treatments. (B) Morphological changes of full-differentiated WAT-derived SVF in response to knockdown of endogenous FGF9 by two shRNAs. (C) mRNA changes of thermogenic biomarkers, adipogenic biomarkers and FGF9 in full-differentiated iWAT-derived SVF under condition of knockdown FGF9. (D) Protein change of UCP1 and PGC1 α in response to FGF9 knockdown. (E and F) Dynamic (E) and quantitative (F) change of OCRs in response to FGF9 knockdown.

effects of FGF9 on browning biomarkers were exhibited as early as the second day after differentiation stage started (Fig. 4A, B, C, D and E), indicating that the involvement of FGF9 in browning regulation occurs in a quite early phase of SVF differentiation. Of note, we observed a significant downregulation of Ebf2, an important molecule for brown adipose maintenance (Rajakumari *et al.* 2013), since 2 days after incubation started, further supporting the early action of FGF9. Thus, we performed an RNA sequencing on the early stage of adipocytes differentiation, including the very beginning (day 0), the first day (day 1) and the second day (day 2) after browning incubation started (Fig. 4F).

By performing unsupervised hierarchical clustering and t-distributed stochastic neighbor embedding analyses to these sequencing data, we observed that samples under different conditions were clearly separated and distributed during the browning process in a time-dependent manner (Fig. 4G and Supplementary Fig. 2A, B, C and D), indicating a highly consistency and comparability of these sequencing data. We observed that the gene expression pattern in response to FGF9 was obviously separated from the control group since the second day after incubation started (Fig. 4G), further supporting the early involvement of FGF9 in the browning process of adipocytes.

We further analyzed the differential genes in these three time points. On day 0, only 22 upregulated genes and 33 downregulated genes were observed (Supplementary Fig. 2B and E), indicating a limited molecular alteration induced by FGF9 in the commitment phase of SVF. Notably, among the increasingly differential genes induced by FGF9 in the subsequent time points (Supplementary Fig. 2C and D), we observed that FGF9 dramatically reversed 146 downregulated genes and 130 upregulated genes on the first day after differentiation started, as well as 338 downregulated genes and 293 upregulated genes on the second day (Fig. 4H). By performing KEGG pathway enrichment analysis, we observed that numerous pathways were clustered on the basis of these reversed genes ($P < 0.05$ by Fisher's exact test) at day 1 and day 2 (Fig. 4I and Supplementary Fig. 2F). Among these altered pathways, HIF1 signaling and cancer-related pathways were consistently upregulated and downregulated, respectively in both of the 2 days (Fig. 4J and Supplementary Fig. 2G). Notably, HIF1 α pathway has been reported as an important participant in adipose metabolism and thermogenic regulation (Jun *et al.* 2017). Thus, we further evaluated the expression of Hif1 α pathway-related genes in these three time points by real-time PCR and observed an anaerobic genes profile,

including upregulation of Glut1 and PDK1, and a possible switch from Cox4i1 to Cox4i2 in response to FGF9 treatment (Fig. 4K), supporting the activation of HIF1 α pathway. These data suggested that FGF9 may inhibit adipose thermogenesis possibly by activating HIF1 α signaling in the early differentiation stage.

FGF9 is highly expressed in adipose tissues of obese humans and mice

Taken together, we demonstrated FGF9 inhibited the browning of white adipocytes, based on which we examined the association of FGF9 with obesity. We compared FGF9 expression between lean and the obese condition and found that the mRNA levels of FGF9 were significantly higher in the subcutaneous adipose tissues of obese human subjects (Fig. 5A and B), ob/ob mice (Fig. 5C and D) and HFD-induced obese mice (Fig. 5E and F). Moreover, we also observed that the increased expression of FGF9 in subcutaneous fat of obese mice mainly originated from its increase in mature adipocyte rather than from SVF cells (Fig. 5G and H). Of note, under normal condition, FGF9, as a paracrine factor that affects the early stage of browning differentiation, expresses higher in mature adipocyte (Fig. 5G and H), indicating a possible interaction between adipose precursor and mature adipocyte in adipose browning regulation. These data indicated that FGF9 may play a potential role in obese development by inhibiting thermogenic capacity of adipose tissue.

Discussion

Browning of WAT, as an adaptive process in response to environmental stresses, has been proven to be able to act as a potential target for preventing obesity and its related metabolic disorders (Bartelt & Heeren 2014); thus, identification of browning-related peptides contributes greatly to treat obesity. In this study, by screening of all FGF family members, we revealed that FGF9 negatively regulates browning of WAT, and increases under obese status, indicating that FGF9 may act as a potential target for treating obesity.

Fibroblast growth factors (FGFs) are a group of signaling proteins with diverse functions in cellular proliferation, survival, differentiation and metabolism (Goetz & Mohammadi 2013, Ornitz & Itoh 2015). Recently, substantial evidence for the involvement of several FGF proteins, such as FGF1, FGF15/19 and FGF21, in glucose metabolism, energy expenditure and adipose

Figure 4

FGF9 suppresses thermogenic genes from the early stage of adipocytes differentiation, following a provocation of HIF1 α pathway. (A, B, C, D and E) Dynamic change of thermogenic genes over the whole process of browning in the presence and absence of FGF9. (F) Schematic design of RNA sequencing to explore underlying mechanism of FGF9, in which 100 ng/mL FGF9 was added in full course over browning process, and the differentiating cells in three time points, including day 0, day 1 and day 2, were collected and undergone sequencing analysis. (G) Cluster images of global sample distribution and relationships analyzed by hierarchical clustering. (H) Heatmap generated using the genes exclusively upregulated by FGF9 treatment in day 1 and day 2 after differentiation initiated. (I) Visualization of the upregulated pathway produced by KEGG enrichment analysis based on the differential genes in Fig. 5H. (J) KEGG enrichment analysis of genes consistently and exclusively upregulated by FGF9 in day 1 and day 2 after differentiation. (K) mRNA level of genes related to HIF1 α pathway in the presence and absence of FGF9 during the browning differentiation process.

Figure 5

Adipose-derived FGF9 expression increases in subcutaneous adipose tissue of obese human and mice. (A and B) mRNA level of FGF9 in subcutaneous adipose tissue (sWAT) and visceral adipose tissue (vWAT) isolated from patients with obesity ($n = 21$) and lean subjects ($n = 11$). (C and D) mRNA level of FGF9 in iWAT and eWAT isolated from ob/ob mice ($n = 11$) and their counterpart control ($n = 11$). (E and F) mRNA level of FGF9 expressed in iWAT and eWAT isolated from mice fed with HFD ($n = 3$) and normal chow ($n = 3$). (G and H) mRNA level of FGF9 expressed in mature adipocyte (AD) and SVF in mice fed with HFD and normal chow.

thermogenesis implied the potential roles of this family in energy homeostasis (Potthoff *et al.* 2011, Fisher *et al.* 2012, Suh *et al.* 2014, Degirolamo *et al.* 2016, Schlein *et al.* 2016). Notably, as two major metabolic-related FGFs, FGF1 and FGF21 have been recently reported as potentially therapeutic targets for metabolic disorders, which are largely due to improved adipose homeostasis (Degirolamo *et al.* 2016, Fisher & Maratos-Flier 2016, Gasser *et al.* 2017). Another member of this family, FGF19, has also showed adipose-related improvement, in which overexpression FGF19 in mice increased BAT mass and thermogenesis (Tomlinson *et al.* 2002). These findings implied an important role of adipose tissues in FGF-induced metabolic improvement. However, few studies

have systematically examined the adipose involvement of FGFs. In this regard, our study provided a systemic expression pattern of FGF family in adipose tissues and found numerous FGFs highly expressed, indicating the potential roles of other FGF members in adipose homeostasis.

FGF9, as a paracrine protein, has been previously reported as an important regulator in various physiological processes, including neurogenesis, sex determination, lung development, nephron progenitor differentiation and so forth (Colvin *et al.* 2001, Bertrand *et al.* 2003, Kim *et al.* 2006, Bowles *et al.* 2010, Barak *et al.* 2012, Small *et al.* 2018). Our previous studies have reported that FGF9 mutation both in human and mice caused multiple synostosis syndrome (SYNS) (Tang *et al.* 2017). However, little is known about its role in adipose tissues. In our study, we found that FGF9 is highly expressed in epididymal WAT and downregulated in the browning of iWAT under cold stress and eWAT under β 3-AR agonist stimulus, indicating a potential involvement of FGF9 in regulating adipose thermogenesis. The different adipose browning depots in response to cold or β 3-AR agonist may due to the he intraperitoneal injection of drug, which is locationally close to epididymal white adipose tissue (eWAT). When we added β 3-AR agonist to SVF cells isolated from epididymal adipose tissue or inguinal adipose tissue *in vitro*, both of them showed a decreased FGF9 level (Supplementary Fig. 1I and J). In contrast to the evident enhancement of browning effect induced by FGF21 (Fisher & Maratos-Flier 2016), FGF9 showed a strong inhibitory effect on the browning of white adipocytes, accompanied with obviously reduced adipogenesis. The inhibition of adipogenic ability induced by FGF9 was determined by Red O oil staining under both overexpression and lower-expression condition, and this inhibitory effect on differentiation is also observed in white adipocyte differentiation, supporting the suppressive role of FGF9 on adipocytes differentiation. Consistently, previous study has reported the stemness-retention effect of FGF9 on metanephric mesenchyme cells, which limits the differentiation potential of pre-matured nephron progenitors (Barak *et al.* 2012). Our data indicated that FGF9 may also participate in maintaining the stemness of adipose progenitor cells to balance the maturation process of adipocytes. Notably, we observed a transit repression of several WNT-related genes, including *Lgr5*, *Wnt16*, *Fzd5*, *Sfrp2*, *Draxin* and *Wisp2*, in response to FGF9 at the very beginning of differentiation phase (Day 0), indicating a possible involvement of Wnt pathway in regulating the early changes caused by FGF9,

but further study is needed to validate this point. To the contrast, however, the alteration of adipogenic genes under condition of Fgf9 knockdown is not as obvious as that in response to high concentration treatment of FGF9 and FGF9 overexpression, the mRNA level of C/ebp α and Pparg shown no alteration between Fgf9-shRNA and control groups, indicating that the concentration of FGF9 secreted endogenously in differentiating SVF may not be sufficient to inhibit adipogenesis. But considering that Fgf9 expresses highly in mature adipocyte (Fig. 5G and H), which is richly contained in adipose tissue *in vivo*, the FGF9 is still possible to play as an inhibitory factor for adipogenesis *in vivo*. On the other hand, the distinct effects between FGF9 and FGF21 may arise from the different signaling pathways affected by the two factors. RNA sequencing analysis revealed a variety of signaling altered in adipocytes under treatment of FGF9, but few of these signaling were the same as FGF21-related pathway in previous studies (Fisher & Maratos-Flier 2016), except for AMPK pathway, which has been reported as downstream pathway of FGF21 in adipocytes (Chau *et al.* 2010). In this study, our data showed a downregulated AMPK pathway in the second day of differentiation, followed by a suppressive effect on browning in the full-differentiated adipocytes. This tendency seems to be consistent with the upregulation of AMPK pathway in response to FGF21 (Chau *et al.* 2010). However, FGF9-induced suppressive tendency on UCP1 appeared on the first day of differentiation, which might suggest an earlier signaling, other than AMPK pathway, mediate FGF9's effects.

By screening those consistently altered pathway in the early phase of adipocytes differentiation in response to FGF9, we focused on HIF1 α pathway for its close relation with adipose homeostasis in previous studies (Trayhurn 2013, 2014). According to these studies, activation of HIF1 α in response to low oxygen supply induced an anaerobic adaptation in adipocytes, including upregulation of PDK1 to inhibit aerobic oxidation of glucose, an increase of GLUT1 expression to elevate glucose supply (Wood *et al.* 2007), and a switch of COX4i1 to COX4i2 to adapt the energy production in hypoxia status (Fukuda *et al.* 2007). These adaptive changes in adipocytes would activate inflammatory response, which contribute to the disorders associated with obesity initiation (Trayhurn 2014). Moreover, overexpression of HIF1 α in adipose tissues suppress the thermogenic effect of BAT and cause obesity (Jun *et al.* 2017), while disruption of HIF1 α and β in adipose tissues causes significant body weight loss with smaller size of white adipocytes and increased glucose

uptake in both BAT and WAT (Jiang *et al.* 2011). These findings demonstrated a negative role of Hif1 α signaling in thermogenic regulation of adipocytes. Consistently, in this study, we observed that FGF9 induced a constant upregulation of Hif1 α expression with corresponding alterations of its downstream molecules at an early stage of adipocytes differentiation. Based on our and other's studies, FGF9 inhibited the browning of white adipocytes possibly through activating Hif1 α signaling which may induce a hypoxia-like environment during differentiation. Future studies are warranted to investigate how FGF9 regulates Hif1 α signaling. Since the significant reduction of brown-related transcriptional factor Pgc1 α , Ebf2, which has been proven as a cooperators of Pparg to activate the transcription of browning genes (Rajakumari *et al.* 2013), how FGF9 transcriptionally interact with these browning genes is still needed to be further explored.

More importantly, we observed that adipose-derived FGF9 increased in obese human and mice, implying a potential role of FGF9 in the development of obesity. According to previous study, thermogenic potential of adipose tissues declined with increased BMI (Virtanen *et al.* 2009), indicating some unknown factors may inhibit thermogenesis under obese condition. In this study, our data showed that FGF9 might partly contribute to this thermogenic decline in obese people.

In conclusion, this study systemically examined the expression of all FGFs in adipose tissues, providing new insights into the physiological function of FGF9 in addition to its pathogenic roles in multiple synostoses syndrome (SYNS). We identified FGF9 as a novel negative regulator for the browning of white adipocytes and associated with mice and human obesity, indicating a potential target for obesity intervention and prevention.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-18-0151>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J W and J H conceived the project and designed the experiments. Y S and R W carried out most of the experiments. S Z, W Li and W Liu assisted in some experiments. L T and Z W contributed valuable comments and advice on the manuscript. W W and G N assisted with statistical analysis. Y S, J W and R L wrote the paper. J W and J H are the guarantors of this work and take responsibility for the integrity of the data analysis.

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