Intraperitoneal administration of follistatin promotes adipocyte browning in high-fat diet-induced obese mice

Haoyu Li¹, Chuanhai Zhang¹, Junyu Liu¹, Wenya Xie¹, Wentao Xu¹,², Fei Liang³, Kunlun Huang¹,², Xiaoyun He¹,²*

¹ Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China, ² Key Laboratory of Safety Assessment of Genetically Modified Organism (Food Safety), Ministry of Agriculture, Beijing, China, ³ Department of Reproductive Physiology, Zhejiang Academy of Medical Sciences, Hangzhou, China

* raininghe@163.com

Abstract

With rapid economic development, the prevalence of obesity has increased remarkably worldwide. Obesity can induce a variety of metabolic diseases, such as atherosclerosis, diabetes, hypertension and coronary heart disease, which significantly endanger the health and welfare of individuals. Brown and beige fat tissues play an important role in thermogenesis in mammals. Recent studies have shown that follistatin (FST) can potentially induce the browning of white adipose tissue (WAT). In this study, high-fat diet-induced obese mice were injected with follistatin for one week to explore the effects of follistatin on browning and metabolism and to determine the mechanism. The results showed that follistatin suppressed obesity caused by a high-fat diet and increased insulin sensitivity, energy expenditure, and subcutaneous fat browning. The beneficial effects remained even after a period of withdrawal. Follistatin promoted secretion of irisin from subcutaneous fat via the AMPK-PGC1α-irisin signal pathway, which induces browning of WAT, and activated the insulin pathway in beige fat thereby promoting metabolism.

Introduction

Brown adipose tissue (BAT) plays an important role in non-shivering thermogenesis, especially in the newborns [1]. BAT is different from WAT with respect to energy metabolism. WAT stores energy mainly in the form of triglycerides during periods of excess energy intake, where BAT releases heat by promoting the consumption of triglycerides and glucose [2]. BAT is characterized by fat accumulation in the form of multilocular lipid droplets, and the presence of numerous mitochondria containing a unique thermogenic protein named uncoupling protein 1 (UCP1). UCP1 is abundantly expressed in the mitochondria of mammalian BAT, and can burn chemical fuels by uncoupling cellular respiration to defend against obesity [3].

When white adipocytes are stimulated by specific factors, such as cold or β3-adrenoceptor, the size of white adipocytes shrinks, the heat production increases, and the adipocytes begin
browning into beige cells [4]. Beige adipose tissue is similar to BAT in morphology and function, as it consists of multilocular lipid droplets and contains a large number of mitochondria. In addition to UCP1, other key BAT-selective genes are highly expressed, such as Prdm16, Pgc1a, and Pparγ. These BAT-selective proteins can promote lipid metabolism and increase the energy expenditure [5, 6]. It is well established that BAT and beige fat can improve insulin sensitivity and decrease body weight in humans and experimental animals. In addition, high levels of the CD137 and TMEM26 proteins are expressed in beige fat, which are key selective markers in beige adipose tissue.

FST is a secreted glycoprotein, which has a high cysteine content. FST has a strong affinity for follicle-stimulating hormone (FSH), activin, and TGF-β superfamily members. FST binds to these proteins in an nearly irreversible manner, making them unable to activate their own receptors [7, 8]. FST is produced in a variety of tissues and organs in adult mammals through autocrine and paracrine mechanisms. Recently, a number of studies have reported that FST can activate BAT and promote the browning of WAT [9, 10]. Myostatin (MST) knockout mice, which were antagonized by FST as a member of TGF-β superfamily, expressed BAT-selective proteins in WAT and showed the appearance of browning [11]. Similarly, in MST knockout mice, the translational levels of the thermogenic genes Ucp1 and Pgc1a, and the transcriptional levels of the beige adipose tissue marker genes Tmem26 and Cd137 were increased [12]. Recently, a number of studies have demonstrated that there were higher levels of fatty acid oxidation and energy metabolism in WAT of MST knockout mice compared to that in wild-type mice [13, 14]. As an inhibitor of MST, exogenous FST increased the expression of thermogenic genes 50%–80% in differentiated preadipocytes. In MST knockout mouse embryonic fibroblasts (MEFs), the expression of the UCP1 protein dropped by 50% compared with wild-type cells [14, 15].

In FST overexpressing mice, BAT mass increased, and the expression of BAT-selective and beige-selective proteins in WAT increased, indicating the formation of beige adipose tissue. At the same time, the expression levels of pp38MAPK/pERK1/2 phosphorylation increased [16]. Inhibition of the pp38MAPK/pERK1/2 pathway in 3T3-L1 cells impeded FST-induced UCP1 protein upregulation, suggesting that FST may induce WAT browning via the pp38MAPK/pERK1/2 pathway. The browning of WAT was observed in MST-knockout mice, and this was shown to be via activation of the AMPK-PGC1α-Fndc5 pathway in muscle [12]. The fibronec- tin type III domain containing 5 (Fndc5), which is the precursor substance of irisin, acts as a muscle factor to promote the expression of brown fat and beige fat-related proteins. As a newly defined myokine, irisin can promote the expression of BAT-selective and beige-selective proteins [17]. In fact, Fndc5 is not only secreted in muscle but also in WAT [18]. As an inhibitor of MST, FST may induce browning of WAT through the AMPK-PGC1α-Fndc5 pathway [19].

At present, there are limited studies of FST in animals, which have used lentiviral vectors to construct an FST-transgenic model [16]. Although this model can stably express high levels of FST in mice for an extended period of time, the method is not suitable for exploring the medicinal value of FST in clinical use. Because FST is an autocrine and paracrine protein which is secreted into the blood by various of tissues and organs in mammals [20], it is feasible to improve blood FST levels within a short time to promote metabolism and regulate blood glucose homeostasis in mice by external injection of FST. In the current study, we show that one week of FST injection can increase metabolic levels and induce subcutaneous WAT browning, possibly by activating AMPK-PGC1α-Fndc5 and pp38MAPK/pERK1/2 pathways, and this effect was observed after stopping the injections for one week. The current work is valuable for the clinical application of FST in activating browning of WAT for the treatment of metabolic syndrome.
Experimental section

Animals

Four- to six-week-old C57BL/6j male mice were obtained from Vital River Laboratories (Beijing). The obesity model was induced using a high-fat diet (HFD), which contained 60% of the energy provided by fat. The experimental design was approved by the Animal Ethics Committee of China Agricultural University, Beijing, and the approval ID of this study was KY20170027. The study was performed in a Specific Pathogen Free animal room of the Supervision, Inspection and Testing Center for Genetically Modified Organisms of the Ministry of Agriculture (Beijing, China; license number SYXK (Beijing) 2015–0045). The animal room was well controlled as the air was exchanged 15 times in an hour, a 12-hour light-dark schedule was maintained, the temperature was controlled at 22 ± 2˚C, and humidity was controlled at 55% ± 10%. The chow fed to experimental animals was purchased from Hufukang biotechnology company (Beijing). The composition and content of the HFD are shown in Table 1. The weight ratio and energy ratio of the experimental diets are shown in Table 2.

FST injection experiment

Before the experiment, all animals were allowed one-week acclimation to adapt to the conditions of the animal room, and then fed with high-fat diet for the next 8 weeks to induce obesity. After high-fat induction, 12 mice with similar body weights were divided into two groups: One group was intraperitoneally injected with 8.5μg/kg bodyweight of Recombinant Mouse Follistatin 288 (R&D systems, 769-FS-025) once a day for one week, while the other group was marked as the control.

FST withdrawal experiment

To explore the persistence of the physiological effect of FST, another 18 high-fat diet-induced obese mice were divided into 3 groups. One group of mice was marked as the follistatin withdrawal group (FSTW group), which was intraperitoneally injected with 8.5μg/kg bodyweight of FST once a day for one week but had no injection treatment for the next week. The second group was intraperitoneally injected with the same volume of phosphate buffered saline (PBS)
once a day for one week, and had no injection treatment for the next week, this was marked as
the PBS withdrawal group (PBSW group). The third group was injected with FST the same as
the first group and when the first week passed, the mice in this group were sacrificed and their
inguinal subcutaneous WAT sampled for Western blotting analysis.

In all experiments, 3 mice in the same group were housed in one cage with free access to
food and water. All animals were fed a high-fat diet and body weight was recorded once a
week. At the end of the experiment, the mice were sacrificed and blood was collected. Inguinal
subcutaneous WAT was fixed using 4% paraformaldehyde and frozen in liquid nitrogen.

Rectal temperature and infrared imaging
The mice were placed in a cold room (4˚C) for 4 h and their rectal temperature was measured
by temperature sensor AT210 (Zhongyi Dapeng, Shenzhen, China). Then, photos of the ani-
mals were taken using a handheld infrared camera FLIR T600 (FLIR, Oregon, US) with a
white board as the background.

Activity and energy expenditure
At the end of the FST withdrawal experiment, the mice were placed in a six-cage activity
meter. After a 12 h acclimation period, activity was measured for 24 h. During the measure-
ment of the activity, the mice had access to the HFD and water.

At the end of the FST withdrawal experiment, the mice were placed in a six-cage oxygen
consumption meter. After a 12 h acclimation period, oxygen consumption ($VO_2$) and carbon
dioxide production ($VCO_2$) were measured for the next 24 h. The data were recorded and the
respiratory exchange ratio (RER) and energy expenditure (EE) were calculated using the for-


| HFD | Weight ratio g% | Energy ratio kcal% |
|-----|----------------|-------------------|
| Protein | 26 | 20 |
| Carbohydrate | 26 | 20 |
| Fat | 35 | 60 |
| total: | | 100 |
| kcal/g | 5.24 |

https://doi.org/10.1371/journal.pone.0220310.t002

Intraperitoneal glucose tolerance test
At the end of the experiment (the FST injection experiment finished in one week, and the FST
withdraw experiment finished in two weeks), an intraperitoneal glucose tolerance test (GTT)
was carried out. All animals were fasted for 16 h and then given an intraperitoneal injection of
glucose (1.5g/kg body weight). Blood glucose levels were detected at 0, 15, 30, 60, and 120 min
after the glucose injection using blood glucose meters (ACCU-CHEK, Performa) and the area
under the curve (AUC) between 0 and 120 min was calculated.

Homeostasis model assessment-insulin resistance
The levels of serum insulin were determined with an insulin ELISA kit (Beyotime, PI602).
Homeostasis model assessment-insulin resistance was calculated using the formula [23]:

$$\text{HOMA-IR} = \frac{\text{fasting insulin (mU/L)} \times \text{fasting glucose (mmol/L)}}{22.5}$$
concentration of blood insulin (μg/L) × concentration of blood glucose (mg/dL)/22.5; the concentration of blood glucose was measured at 0 min in the GTT.

**Hematoxylin and eosin staining and immunohistochemistry**

The subcutaneous adipose tissue fixed in 4% paraformaldehyde was cut to 1.0 cm × 1.0 cm × 0.3 cm cakes and embedded in paraffin. Each embedded tissue was then sliced to 5 μm thickness, placed on slides, and stained by hematoxylin and eosin (HE). The cell morphology of subcutaneous adipose was observed under the microscope. For immunohistochemistry (IHC) staining, tissue sections were incubated with blocking buffer for 1 h after antigen retrieval. Then, the tissue sections were incubated overnight with diluent containing Ucp1 primary antibodies. After washing with TBST, the samples were incubated for 60 min with diluent containing secondary antibodies. Then, the tissue sections were washed and reacted with ECL reagent.

**Real time-qPCR analysis**

Total RNA was extracted using Trizol. Frozen subcutaneous adipose tissue (0.1 g) was added into 1 ml Trizol and a handheld homogenizer was used to break up the tissue. Samples were centrifuged for 10 min at 12,000 g, 0.2 ml chloroform was added to the supernatant, and samples were centrifuged for 15 min at 12,000 g. The supernatant was then mixed with 0.5 ml isopropanol and centrifuged 10 min at 12,000 g. The sedimentation after centrifugation contained total RNA, which was collected for further analysis.

The inverse transcription of RNA was performed using the One Step gDNA removal and cDNA Synthesis Supermix kit (Transgen Biotech, AT311). The real time-qPCR (RT-qPCR) was carried out using the Green qPCR Supermix kit (Transgen Biotech, AQ101). The cyclophilin gene was used as an internal control to quantify the expression of the BAT maker genes and beige maker genes and all primers were synthesized by the Beijing Ruibiotech company. The primers used are shown in Table 3 [24–26].

**Western blotting**

Total protein from subcutaneous adipose was extracted using RIPA lysis buffer containing 1 mM PMSF. The homogeneous mixture was centrifuged at 10,000 g and the protein concentration of the supernatant was detected using a BCA kit (Beyotime Biotechnology, P0011). The protein solution was then mixed with loading buffer and heated for 5 min in boiling water.

An equal amount of each protein sample was loaded and separated by 12.5% SDS PAGE in an electrophoresis tank. Then, the protein of sample was transferred from the gel to a PVDF membrane. The PVDF membrane carrying the protein was blocked with 5% BSA. Western blotting was carried out using corresponding detection antibodies.

**Statistical analysis**

Data was presented as mean ± SEM, and differences between groups were analyzed by Student’s t-tests using GraphPad Prism 5.0. If p value of T-test was less than 0.05, the difference was considered as significant.

**Results**

**FST injection decreased body weight in obese mice**

In the FST injection experiment, the high-fat diet-induced obese mice were intraperitoneally injected with FST once a day for one week. The body weight and body fat index were measured at the beginning and end of the experiment. The mice injected with FST had a lower body
weight than the control group, which had no FST treatment (Fig 1A), and the body fat indexes of the FST injected mice were significantly lower than controls (Fig 1B). In addition, there was no significant difference in food intake, which was approximately 2 g high-fat chow per day, between the two groups (Fig 1C). The data suggest that FST injection can decrease body weight via a biological mechanism that does not inhibit food intake.

**FST injection improved glucose metabolism in obese mice**

There was no significant difference in blood glucose between the two groups at 0 min. After glucose injection, the blood glucose of FST injected mice was significantly lower than that of the control group at 15 min, 30 min, 60 min, 90 min (Fig 1D). The AUC of FST injected mice was significantly lower than the controls (Fig 1E). Compared to the control group, the mice injected with FST had a significantly lower HOMA-IR value (Fig 1F), which suggested that FST may be used to treat insulin resistance caused by obesity.

**FST injection improved thermogenesis in obese mice**

After 4 h treatment at 4˚C, the rectal temperature of the control group was approximately 36˚C, while the rectal temperature of the FST injection group was approximately 37.5˚C, which was significantly higher than the controls (Fig 1G). From the infrared imaging, the high-temperature area of the injection group was higher than that of the control group (Fig 1H). These results indicated that FST injection promoted thermogenesis in mice and allowed the maintenance of a higher body temperature under cold conditions.

**FST injection induced browning of subcutaneous adipocytes**

By observing the H&E stained slides of the inguinal adipose under a microscope, it was determined that the size of subcutaneous adipocytes in FST injected mice was significantly smaller

---

***Table 3. Primer sequences.***

| Primer Names | Primer Sequences |
|--------------|------------------|
| Prdm16-F     | GAAGTCACAGGAGGACACGG |
| Prdm16-R     | CTGCTCTTCAACACCTCC |
| CyclophiA-F  | CAAATGCTGGACCAAACA |
| CyclophiA-R  | GGCATCCAGCCATTCGTC |
| UCP1-F       | GGCACAAAGAAGGATTCG |
| UCP1-R       | TAAAGCGCCGTGATCCGT |
| PGC-1a-F     | ACAGCCTTGTGGGATTTG |
| PGC-1a-R     | TGGAGGGCTGAGCAATTTT |
| Tmem26-F     | ACCCTGTCAATCCCACAGG |
| Tmem26-R     | TGGTGTGGGATGGCTAAGT |
| CD137-F      | CGTGCAGAATCTTCGGATAAC |
| CD137-R      | GCCACCTATGCTGGAGAAGG |
| PPARγ-F      | TCGCTGATGCAGCTGCTATG |
| PPARγ-R      | GAGAGGTCCACAGGTCATT |
| COX2-F       | CTGGTGCTAATACGACTGCT |
| COX2-R       | GACGGTACTGGTGGGAGAA |
| LHX8-F       | GAGCTGATAGTGCGAGAGA |
| LHX8-R       | GTGAGCGTCTTGGCTCCCAG |
| Zic1-F       | CTGGTCAACACATCCGAGT |
| Zic1-R       | TTGCAAGAGGCTGGCTGTC |

[Hyperlink to Table 3]
than in the controls, and multilocal lipid droplet structures appeared in the injection group (Fig 2A). Immunohistochemistry staining showed that the UCP1 signal in the FST group was stronger than the Control group (Fig 2B).

The expression levels of *Ucp1*, *Prdm16*, *Pgc1α* and *Pparγ* (the BAT maker genes involved in thermogenesis) in the injection group were significantly higher than in the control group (Fig
The expression of *Tmem26* and *Cd137*, two marker genes of beige fat, in the injection group were significantly higher than in the control group (Fig 2D). Combined with characteristic gene expression and cell morphology, it can be concluded that FST injection induces subcutaneous WAT browning.

**Persistence of FST on physiological functions**

Analysis of the pathological sections of the inguinal adipocyte tissue of mice showed that the size of subcutaneous adipocytes after stopping the drug was obviously smaller than the controls, which were injected with PBS for one week (Fig 3A). The expression of BAT markers and beige markers in subcutaneous adipocytes was detected by RT-qPCR and calculated using Cyclophilin as an internal control. The results showed that the expression of *Ucp1*, *Pgc1α*, *Prdm16*, *Pparγ* and beige markers including *Tmem26* and *Cd137*, injection of FST significantly increased the expression of these genes in the subcutaneous fat of DIO mice. Bars represent the mean ± SEM, n = 6. *P < 0.05* and **P < 0.01* when the FST injection group was compared with the control group. Abbreviations: FST, the FST injection group, which received one week of FST. Control, the control group, which had no treatment in the FST injection experiment.

![Fig 2](https://doi.org/10.1371/journal.pone.0220310.g002)

2C). The expression of *Tmem26* and *Cd137*, two marker genes of beige fat, in the injection group were significantly higher than in the control group (Fig 2D). Combined with characteristic gene expression and cell morphology, it can be concluded that FST injection induces subcutaneous WAT browning.

**Persistence of FST on physiological functions**

Analysis of the pathological sections of the inguinal adipocyte tissue of mice showed that the size of subcutaneous adipocytes after stopping the drug was obviously smaller than the controls, which were injected with PBS for one week (Fig 3A). The expression of BAT markers and beige markers in subcutaneous adipocytes was detected by RT-qPCR and calculated using Cyclophilin as an internal control. The results showed that the expression of *Ucp1*, *Pgc1α*, *Prdm16*, *Pparγ* and beige markers including *Tmem26* and *Cd137*, injection of FST significantly increased the expression of these genes in the subcutaneous fat of DIO mice. Bars represent the mean ± SEM, n = 6. *P < 0.05* and **P < 0.01* when the FST injection group was compared with the control group. Abbreviations: FST, the FST injection group, which received one week of FST. Control, the control group, which had no treatment in the FST injection experiment.

**Persistence of FST on physiological functions**

Analysis of the pathological sections of the inguinal adipocyte tissue of mice showed that the size of subcutaneous adipocytes after stopping the drug was obviously smaller than the controls, which were injected with PBS for one week (Fig 3A). The expression of BAT markers and beige markers in subcutaneous adipocytes was detected by RT-qPCR and calculated using Cyclophilin as an internal control. The results showed that the expression of *Ucp1*, *Pgc1α*, *Prdm16*, *Pparγ* and beige markers including *Tmem26* and *Cd137*, injection of FST significantly increased the expression of these genes in the subcutaneous fat of DIO mice. Bars represent the mean ± SEM, n = 6. *P < 0.05* and **P < 0.01* when the FST injection group was compared with the control group. Abbreviations: FST, the FST injection group, which received one week of FST. Control, the control group, which had no treatment in the FST injection experiment.

**Persistence of FST on physiological functions**

Analysis of the pathological sections of the inguinal adipocyte tissue of mice showed that the size of subcutaneous adipocytes after stopping the drug was obviously smaller than the controls, which were injected with PBS for one week (Fig 3A). The expression of BAT markers and beige markers in subcutaneous adipocytes was detected by RT-qPCR and calculated using Cyclophilin as an internal control. The results showed that the expression of *Ucp1*, *Pgc1α*, *Prdm16*, *Pparγ* and beige markers including *Tmem26* and *Cd137*, injection of FST significantly increased the expression of these genes in the subcutaneous fat of DIO mice. Bars represent the mean ± SEM, n = 6. *P < 0.05* and **P < 0.01* when the FST injection group was compared with the control group. Abbreviations: FST, the FST injection group, which received one week of FST. Control, the control group, which had no treatment in the FST injection experiment.

To investigate the persistence of the physiological effects of FST, the FST withdrawal experiment was performed. The body fat rate of the mice in the FST withdrawal group was significantly lower than in the PBS withdrawal group (Fig 3D), even though the mice were still on a high-fat diet.
Fig 3. Persistence of FST on physiological function. A, hematoxylin and eosin staining of inguinal subcutaneous WAT taken from the FST withdrawal and PBS withdrawal groups. One week after FST withdrawal, the size of adipocytes in the FSTW group was still significantly smaller than in the PBSW group. B and C, the expression of BAT markers including Ucp1, Pgc1α, Prdm16, Ppary and Beige markers including Tmem26 and Cd137. After FST withdrawal, these key genes for the browning of WAT still maintained a high level of expression compared with the PBSW group. D, the body fat index of the FSTW group was significantly lower than in the PBSW group after FST withdrawal for one week. E and F, GTT and area under the curve of GTT, the level of blood glucose metabolism in the FSTW group was significantly higher than in the PBSW group one week after FST injection was withdrawn. G and H, rectal temperature and infrared imaging of mice in a 4°C environment. After stopping the FST treatment, the FSTW group still maintained a higher body temperature in a cold environment compared with the PBSW group, which served as the control. I and J, the energy expenditure and activity of mice in the FST withdrawal experiment. Bars represent the mean ± SEM, n = 6. *P < 0.05 and **P < 0.01 when the FST injection group was compared with the control. Abbreviations: FSTW (follistatin withdrawal), the group received one week of FST injections with no injections for the next week. PBSW (PBS withdrawal), the group received PBS injections for one week and no PBS injections for the next week.

https://doi.org/10.1371/journal.pone.0220310.g003
After the withdrawal of FST, a GTT was performed on the two groups. There was no significant difference in blood glucose between the groups at 0 min. At 15 min, 30 min, 90 min, and 120 min after glucose injection, the blood glucose of the mice in the FST withdrawal group was significantly lower than in the PBS withdrawal group (Fig 3E). The AUC results showed that the AUC values in the withdrawal group were significantly lower than the control either (Fig 3F).

After cold stimulation, the rectal temperature of the FST withdrawal group (about 37.5˚C) was significantly higher than the control (about 36˚C, Fig 3G). The high-temperature area in the FST withdrawal group was greater than in the controls (Fig 3H). To accurately measure the energy metabolism of the mice, the activity and oxygen consumption of the FSTW and the PBSW groups was measured using metabolic cages. The EE of the FSTW group was significantly higher than in the PBS group (Fig 3I), although there was no significant difference in activity (Fig 3I).

Overall, FST increased the metabolic level and reduced the body weight of DIO mice, and this effect was maintained one week after cessation of FST. Considering that the inguinal subcutaneous fat was still beige fat after withdrawing FST, our data suggested that FST injection increased energy expenditure by promoting the browning of WAT.

**FST induced browning by promoting irisin secretion in beige adipose**

The expression of AMPK, p-AMPK, PGC1α and FNDC5 in the FST withdrawal group was significantly higher than in the PBS withdrawal group (Fig 4A and 4B), and the FNDC5 protein level was 2.7-fold higher ($p < 0.01$, Fig 4A and 4B). It is worth noting that there was no significant difference in the four proteins between the FST group and the FST withdrawal group (Fig 4A and 4B). These results suggested that FST may induce browning of subcutaneous adipose by promoting the secretion of irisin in subcutaneous adipose. In addition, although the expression levels of AMPK and p-AMPK increased significantly after injection of FST, the phosphorylation level of AMPK did not change significantly (Fig 4C).

To further verify that FST induced subcutaneous fat browning through the irisin signaling pathway, the phosphorylation levels of ERK1/2, the downstream signal of irisin, was detected. The phosphorylation level of ERK1/2 pathway proteins in the FST withdrawal group was increased by 2.11-fold ($p < 0.05$) compared to the PBS withdrawal group (Fig 4D and 4E). Interestingly, there was no significant difference between the FST and FST withdrawal groups (Fig 4D and 4E).

**FST promoted blood glucose metabolism by activating the insulin signaling pathway in beige adipose**

The expression IR, AKT, P-AKT, and GLUT4, four key proteins in the insulin signaling pathway, were measured in the FST, FST withdrawal and PBS withdrawal groups.

The expression of key proteins in the insulin signaling pathway in the FST withdrawal group was significantly higher than in the PBS withdrawal group. The expression of IR was increased by 2.5-fold ($p < 0.01$), AKT was increased by 3.4-fold ($p < 0.01$), P-AKT was increased by 3.0-fold ($p < 0.01$) and Glut4 was increased by 2.4-fold ($p < 0.01$) compared to PBS withdrawal group. Except for the AKT protein, there was no significant difference between the FST group and the FST withdrawal group in the insulin signaling pathway proteins (Fig 4F and 4G).

**FST promotes mitochondrial heat production in adipocytes**

The increased thermogenesis in mice injected with FST was associated with mitochondrial activity in beige fat. The results of western blotting showed that the expression of key proteins
in the mitochondria of the FST withdrawal group was significantly higher than in PBS withdrawal group. The expression of UCP1 was increased by 2.1-fold (p < 0.01) compared to the PBS withdrawal group. The increase in expression of UCP1 and OXPHOS including ATP5A, UQCRC2 and SDHB, suggested that the level of phosphorylation and uncoupling in mitochondria was promoted by FST. There was no significant difference between FST group and FST withdrawal group on these mitochondrial proteins, which resulted in an intensification of energy metabolism in the mice (Fig 4H and 4I).

**Discussion**

Browning of WAT, as one of the important biological functions of FST, has been a focus of current research. FST injection groups exhibited a browning phenotype with respect to cell morphology after FST injection, and the size of the adipocytes was significantly reduced. The heat-producing function of beige fat and brown fat is largely dependent on the uncoupling
effect of UCP1, a typical brown fat characteristic gene. It is an important reference for determining adipose browning. In this experiment, the expression of Ucp1, Prdm16, Pgc1α, and Pparγ was significantly increased after injection of FST. Aside from the brown fat characteristic genes, the beige fat characteristic genes are an important criterion for judging WAT browning. Garron Dodd et al. used leptin and insulin to promote fat browning, and found that adipocytes shrank and the expression of brown markers and beige markers increased. J Lin et al. also showed similar results in experiments with Tibetan pigs, in which the browning was attributed to UCP3 which is an uncoupling protein similar to UCP1 [27, 28]. In our study, the expression of Tmem26 and Cd137, two beige markers, increased after injection of FST, indicating that FST injection induced inguinal subcutaneous WAT browning in obese mice.

Obesity caused by a high-fat diet was significantly suppressed following injection with FST. The body weights and body fat indexes of mice injected with FST were significantly reduced without changing food intake. S Fang et al. used FXR agonists to induce browning of WAT in mice. Their results showed a similar phenotype as in the current study, in that there was weight loss after WAT browning in mice [29]. Taken together, these results suggest that FST may inhibit obesity through beige fat generation.

One of the important functions of beige fat is to promote heat production and increase energy metabolism. After the browning of subcutaneous adipose, the WAT (which serves as an energy storage tissue) became beige adipose tissue (which serves as an energy consumption tissue and is directly related to the activation of mitochondrial function and an increase in the number of mitochondria in adipose) [30]. UCP1, the core protein of uncoupling, is key to the function of brown fat and beige fat, and plays an important role in energy release [31]. In the UCP1 overexpressing transgenic mouse model, mice were resistant to obesity induced by a high-fat diet [32]. Pontus et al. successfully constructed a mouse model which overexpressed PGC1α. The transgenic mice showed browning of subcutaneous WAT, and UCP1 protein content was significantly increased in browned beige fat, results that are similar to the current experiment using FST injection [33]. These results indicated that FST promoted thermogenesis by promoting the expression of UCP1 in beige fat and increasing the uncoupling level of mitochondria. In FST knockout mouse embryonic fibroblasts, cell oxygen consumption was significantly lower than in wild-type mouse embryonic fibroblasts, and beige fat exhibited considerable heat production due to the large amount of UCP1. [30, 34]. In the current experiment, the thermogenesis of mice was increased after FST injection (Fig 1G and 1H), and the expression of the Ucp1 gene in beige fat was increased (Fig 2C), indicating that FST resists obesity by promoting energy metabolism in beige fat.

Type 2 diabetes is a classic metabolic disease caused by obesity. In this study, after the injection of FST, blood glucose tolerance increased and insulin resistance decreased, indicating that FST improved blood glucose metabolism in obese mice. Many experiments have shown that when MST is inhibited, adipocytes are browned. As an antagonist of MST, FST can promote the expression of the PGC1α gene, a classical BAT marker, when it acts on adipose cells [30, 35]. In the MST knockdown pig model constructed by Chunbo Cai et al. and the PGC1α overexpressed mouse model constructed by Pontus Bostrom et al., insulin sensitivity in experimental animals was up-regulated with fat browning [33, 36]. In experiments on blood glucose metabolism, studies have generally focused on the muscles. In the gastrocnemius muscle of MST knock out Meishan pigs, the expression level of the insulin signaling pathway IR-AKT-GLUT4 was significantly increased compared to that of wild type pigs [36]. Beige fat, as an energy metabolism tissue, also plays an important role in blood glucose metabolism. In our study, western blot analysis of subcutaneous fat revealed that the insulin signaling pathway proteins in beige fat were significantly improved (Fig 4F and 4G), indicating that FST can regulate blood glucose by activating the insulin pathway in fat as well as muscle.
Irisin is a recently discovered muscle factor which promotes WAT browning and regulates blood glucose. Blood irisin content is sharply increased after vigorous exercise, suggesting that exercise promotes skeletal muscle secretion of irisin. In the myocytes of MST knock out mice, the amount of irisin secretion was significantly higher than in the wild type mice. In the experiments on MST knockout animal models performed by Chunbo Cai et al., MST deletion promoted the secretion of irisin via the AMPK-PGC1α-FNDC5 signaling pathway [36]. Roca-Rivada et al. found that adipocytes also secrete irisin, suggesting that similar to muscles, the irisin secretion may be present in adipose tissue [37]. In the current experiment, the AMPK-PGC1α-FNDC5 pathway in beige fat was verified. The protein expression level in the FST withdrawal group was significantly higher than in the PBS withdrawal control group. This suggested that FST regulated the synthesis of irisin in beige fat through promoting AMPK and PGC1α expression (Fig 4A and 4B). Yuan Zhang et al. reported that irisin promoted the browning of 3T3L1 adipocytes when U0126 inhibitors were used to block the ERK1/2 phosphorylation pathway. In this experiment, adipocytes did not turn beige, suggesting that irisin induces browning via the ERK1/2 signal pathway. The results of the current experiment showed that the phosphorylation levels of ERK1/2 signaling pathway proteins was significantly increased after FST injection in obese mice. It can be speculated that after the FST injection, the secretion of irisin is promoted by the AMPK-PGC1α-FNDC5 pathway in muscle and fat. Irisin induces browning of subcutaneous fat by promoting the phosphorylation levels of ERK1/2 (Fig 4A–4E). Other studies have shown that the concentration of blood irisin is inversely proportional to insulin sensitivity, that is, when insulin resistance occurs in the body, the blood concentration of irisin is also significantly increased. This indicates that FST can increase insulin sensitivity by promoting the secretion of irisin.

Many different drugs are available and induce effect of weight reduction via suppressing appetite, promoting excretion, and hindering absorption, such as bupropion, L-carnitine, and orlistat [38–40]. Due to the influence of drug mechanisms, most drugs cause side effects such as diarrhea, fecal incontinence, depression, or addiction. People must also continue to take the medicines to maintain weight loss, and a rebound in weight gain can also occur if people withdraw the medicines. Therefore, most of these small molecules have been banned [41]. In the FST withdrawal experiment, after one week of FST withdrawal, the effect of FST on the inhibition in fat increase, promotion of body heat production, and acceleration of blood glucose metabolism did not disappear (Fig 3D–3J), indicating that the effects of FST are persistent.

FST injection increased the metabolism of beige fat, and the expression of AKT was slightly decreased. Except for AKT, the expression of insulin pathway proteins did not change significantly after cessation of FST treatment (Fig 4F and 4G). FST promoted expression of the UCP1 and OXPHOS proteins in the mitochondria of beige fat, which aggravated the uncoupling and oxidative phosphorylation of adipocytes. In addition, the relative expression of UCP1 and OXPHOS did not decrease significantly after one week of cessation of FST injection. This may explain how metabolism promotion in obese mice can still exist even if the FST is withdrawn.

**Conclusions**

Due to the development of social productivity, obesity and related metabolic diseases induced by high-fat diets and a sedentary lifestyle are plaguing people at a rapid pace. Our study suggests that FST, an autocrine and paracrine protein, has great potential in the treatment of obesity and metabolic syndrome by inducing the browning of white fat and promoting glucose and lipid metabolism. FST may therefore be developed as a new drug for the treatment of obesity. However, further evaluation of safety and the physiological actions of FST require more exploration.
Acknowledgments
We acknowledge the major special projects on the cultivation of genetically modified organisms. We thank Guishan Li, Jianbing Ge and Jia Xu for helping dissect mice in the experiments.

Author Contributions
Conceptualization: Chuanhai Zhang, Xiaoyun He.
Data curation: Haoyu Li, Junyu Liu, Wenya Xie.
Formal analysis: Haoyu Li.
Funding acquisition: Fei Liang, Xiaoyun He.
Investigation: Haoyu Li.
Methodology: Xiaoyun He.
Resources: Kunlun Huang.
Software: Chuanhai Zhang.
Validation: Wentao Xu.
Writing – original draft: Haoyu Li.
Writing – review & editing: Haoyu Li, Xiaoyun He.

References
1. Kajimura S, Seale P, Spiegelman BM. Transcriptional control of brown fat development. Cell Metabolism. 2010; 11(4):257. https://doi.org/10.1016/j.cmet.2010.03.005 PMID: 20374957
2. Bartelt A, Bruns OT, Reimer R, Hohenberg H, Ittrich H, Peldschus K, et al. Brown adipose tissue activity controls triglyceride clearance. Nature Medicine. 2011; 17(2):200. https://doi.org/10.1038/nm.2297 PMID: 21258337
3. Nedergaard J, Bengtsson T, Cannon B. New Powers of Brown Fat: Fighting the Metabolic Syndrome. Cell Metabolism. 2011; 13(3):238. https://doi.org/10.1016/j.cmet.2011.02.009 PMID: 21356513
4. Vitali A, Murano I, Zingaretti MC, Frontini A, Ricquier D, Cinti S. The adipose organ of obesity-prone C57BL/6J mice is composed of mixed white and brown adipocytes. Journal of Lipid Research. 2012; 53(4):619. https://doi.org/10.1194/jlr.M018846 PMID: 22271685
5. Kajimura S, Saito M. A New Era in Brown Adipose Tissue Biology: Molecular Control of Brown Fat Development and Energy Homeostasis. Annual Review of Physiology. 2014; 76:225.
6. Long JZ, Svensson KJ, Tsai L, Zeng X, Roh HC, Kong X, et al. A smooth muscle-like origin for beige adipocytes. Cell Metabolism. 2014; 19(5):810–20. https://doi.org/10.1016/j.cmet.2014.03.025 PMID: 24709624
7. Nakamura T, Takio K, Eto Y, Shibai H, Titani K, Sugino H. Activin-binding protein from rat ovary is follistatin. Science. 1990; 247(4944):836–9. https://doi.org/10.1126/science.2106159 PMID: 2106159
8. Phillips DJ. Regulation of activin’s access to the cell: why is mother nature such a control freak? Bioessays. 2000; 22(8):689–96. https://doi.org/10.1002/1521-1878(200008)22:8<689::aid-bies2>3.0.CO;2-5 PMID: 10918299
9. Singh R, Braga M, Reddy ST, Lee SJ, Parveen M, Grijalva V, et al. Follistatin targets distinct pathways to promote brown adipocyte characteristics in brown and white adipose tissues. Endocrinology. 2017; 158(5):1217–30. https://doi.org/10.1210/en.2016-1807 PMID: 28324027
10. Melissa B, Reddy ST, Laurent V, Shehla P, Victor G, David S, et al. Follistatin promotes adipocyte differentiation, browning, and energy metabolism. Journal of Lipid Research. 2014; 55(3):375–84. https://doi.org/10.1194/jlr.M039719 PMID: 24443561
11. Braga M, Pervin S, Norris K, Bhasin S, Singh R. Inhibition of in vitro and in vivo brown fat differentiation program by myostatin. Obesity. 2013; 21(6):1180–8. https://doi.org/10.1002/oby.20117 PMID: 23868854
12. Shan T, Liang X, Bi P, Kuang S. Myostatin knockout drives browning of white adipose tissue through activating the AMPK-PGC1α-Fndc5 pathway in muscle. The FASEB Journal. 2013; 27(5):1981–9. https://doi.org/10.1096/fj.12-225755 PMID: 23362117

13. Choi SJ, Yablonkareveni Z, Kaiyala KJ, Ogimoto K, Schwartz MW, Wisse BE. Increased energy expenditure and leptin sensitivity account for low fat mass in myostatin-deficient mice. Am J Physiol Endocrinol Metab. 2011; 300(6):1031–7.

14. Matzuk MM, Lu N, Vogel H, Sellheyer K, Roop DR, Bradley A. Multiple defects and perinatal death in mice deficient in follistatin. Nature. 1995; 374(6520):360–3. https://doi.org/10.1038/374360a0 PMID: 7885475

15. Braga M, Reddy ST, Vergnes L, Pervin S, Grijalva V, Stout D, et al. Follistatin promotes adipocyte differentiation, browning, and energy metabolism. Journal of Lipid Research. 2014; 55(3):375–84. https://doi.org/10.1194/jlr.M039719 PMID: 24443561

16. Singh R, Braga M, Reddy ST, Lee SJ, Parveen M, Grijalva V, et al. Follistatin targets distinct pathways to promote brown adipocyte characteristics in brown and white adipose tissues. Endocrinology. 2017.

17. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, et al. A PGC1α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature. 2012; 481(7382):463–8. https://doi.org/10.1038/nature10777 PMID: 22237023

18. Arturo RR, Cecilia C, Senin LL, Landrovo MO, Javier B, Ana BC, et al. FNDC5/irisin is not only a myokine but also an adipokine. Plos One. 2013; 8(4):e60563. https://doi.org/10.1371/journal.pone.0060563 PMID: 23593248

19. Triantafyllou GA, Skouvakalidou EC, Saridakis ZG, Kyngopoulos G, Dima DI, Apostolou A, et al. Circulating Follistatin and Irisin in Young, Healthy Individuals: A One-Year Prospective Cohort Study. GPCRs, Growth Factors, Tyrosine Kinases, Inhibits, Activins, and TGF Beta Superfamily (posters): Endocrine Society; 2016. p. SAT-123-SAT-.

20. Braga M, Reddy ST, Vergnes L, Pervin S, Grijalva V, Stout D, et al. Follistatin promotes adipocyte differentiation, browning, and energy metabolism. Journal of Lipid Research. 2014; 55(3):375. https://doi.org/10.1194/jlr.M039719 PMID: 24443561

21. Xu J, Lloyd D, C, Stanislaus S, Chen M, Sivits G, Vonderfecht S, et al. Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. Diabetes. 2009; 58(1):250–9. https://doi.org/10.2337/db08-0392 PMID: 18840786

22. Stewart LK, Soileau JL, Ribnicky D, Wang ZQ, Raskin I, Poulev A, et al. Quercetin transiently increases energy expenditure but persistently decreases circulating markers of inflammation in C57BL/6J mice fed a high-fat diet. Metabolism Clinical & Experimental. 2008; 57(7 Suppl 1):S39.

23. Ding S, Chi MM, Scull BP, Rigby R, Schwerbrock NMJ, Magness S, et al. High-Fat Diet: Bacterium Interactions Promote Intestinal Inflammation Which Precedes and Correlates with Obesity and Insulin Resistance in Mouse. Plos One. 2010; 5(8):e12191. https://doi.org/10.1371/journal.pone.0012191 PMID: 20808947

24. Shan T, Liang X, Bi P, Kuang S. Myostatin knockout drives browning of white adipose tissue through activating the AMPK-PGC1α-Fndc5 pathway in muscle. Faseb Journal Official Publication of the Federation of American Societies for Experimental Biology. 2013; 27(5):1981. https://doi.org/10.1096/fj.12-225755 PMID: 23362117

25. Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, et al. A PGC1α-dependent myokine that drives browning of white fat and thermogenesis. Nature. 2012; 481(7382):463–8. https://doi.org/10.1038/nature10777 PMID: 22237023

26. Singh R, Braga M, Reddy ST, Lee SJ, Parveen M, Grijalva V, et al. Follistatin targets distinct pathways to promote brown adipocyte characteristics in brown and white adipose tissues. Endocrinology. 2017; 158(5).

27. Dodd GT, Decherf S, Loh K, Simonds SE, Wiede F, Ballard E, et al. Leptin and Insulin Act on POMC Neurons to Promote the Browning of White Fat. Cell. 2015; 160(1–2):88–104. https://doi.org/10.1016/j.cell.2014.12.022 PMID: 2594176

28. Lin J, Cao C, Tao C, Ye R, Dong M, Zheng Q, et al. Cold adaptation in pigs depends on UCP3 in beige adipocytes. Journal of Molecular Cell Biology. 2017; 9(5):364. https://doi.org/10.1093/jmcb/mjx018 PMID: 28486865

29. Fang S, Suh JM, Reilly SM, Yu E, Osborn O, Lackey D, et al. Intestinal FXR agonism promotes adipose tissue browning and reduces obesity and insulin resistance. Nature Medicine. 2015; 21(2):159. https://doi.org/10.1038/nm.3760 PMID: 2559344

30. Rajan S, Melissa B, Shehla P. Regulation of brown adipocyte metabolism by myostatin/follistatin signaling. Frontiers in Cell & Developmental Biology. 2014; 2(2):60.
31. Nedergaard J, Bengtsson T, Cannon B. New Powers of Brown Fat: Fighting the Metabolic Syndrome. Cell Metabolism. 2011; 13(3):238–40. https://doi.org/10.1016/j.cmet.2011.02.009 PMID: 21356513
32. Hansen JB, Kristiansen K. Regulatory circuits controlling white versus brown adipocyte differentiation. Biochemical Journal. 2006; 398(2):153–68. https://doi.org/10.1042/BJ20060402 PMID: 16898874
33. Boström P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, et al. A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature. 2012; 481(7382):463–8. https://doi.org/10.1038/nature10777 PMID: 22237023
34. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, Ishibashi J, et al. Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. Journal of Clinical Investigation. 2010; 121(1):96. https://doi.org/10.1172/JCI44271 PMID: 21123942
35. Melissa B, Shehla P, Keith N, Shalender B, Rajan S. Inhibition of in vitro and in vivo brown fat differentiation program by myostatin. Obesity. 2013; 21(6):1180–8. https://doi.org/10.1002/oby.20117 PMID: 23868854
36. Cai C, Qian L, Jiang S, Sun Y, Wang Q, Ma D, et al. Loss-of-function myostatin mutation increases insulin sensitivity and browning of white fat in Meishan pigs. Oncotarget. 2017; 8(21):34911–22. https://doi.org/10.18632/oncotarget.16822 PMID: 28432282
37. Roca-Rivada A, Castelao C, Senin LL, Landrove MO, Baltar J, Belén CA, et al. FNDC5/irisin is not only a myokine but also an adipokine. Plos One. 2013; 8(4):e60563. https://doi.org/10.1371/journal.pone.0060563 PMID: 23593248
38. Kim KK, Suh HS, Hwang IC, Ko KD. Influence of eating behaviors on short-term weight loss by orlistat and anorectic agent. Eating Behaviors. 2014; 15(1):87–90. https://doi.org/10.1016/j.eatbeh.2013.10.019 PMID: 24411757
39. Miller LE. Lorcaserin for Weight Loss: Insights Into US Food and Drug Administration Approval. Journal of the Academy of Nutrition & Dietetics. 2013; 113(1):25–30.
40. Odo S, Tanabe K, Yamauchi M. A Pilot Clinical Trial on L-Carnitine Supplementation in Combination with Motivation Training: Effects on Weight Management in Healthy Volunteers. Food & Nutrition Sciences. 2013; 4(2):222–31.
41. Martínez-Sánchez N, Alvarez CV, Ferne J, Nogueiras R, Diéquez C, López M. Hypothalamic effects of thyroid hormones on metabolism. Best Practice & Research Clinical Endocrinology & Metabolism. 2014; 28(5):703–12.