International Journal of Sciences

Research Article Volume 8 – March 2019 (03)

Transgenic n-3 PUFAs Enrichment Regulates Related Physiological Activities by Affecting Nerves

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Abstract: Obesity is generally defined as an excess of body fats and related to weight gain. The hypothalamic arcuate nucleus (ARC) is responsible for regulating peripheral signals that control food intake and energy balance. Uncoupling protein-1 (*UCP-1*) and *UCP-2* are related-genes that adjust body weight. The aim of this study is to explore the effect of *fat-1* gene on body weight. We found that the weight/length ratios of *fat-1* transgenic mice were smaller than wild-type (WT) mice. We hypothesized that an increase in the levels of n-3 PUFAs might alter the expression of hypothalamic neuropeptide and lead to weight loss in *fat-1* mice because *fat-1* gene transforms n-6 polyunsaturated fatty acids (PUFAs) to n-3 PUFAs. Therefore, the appetite-regulating neuropeptides in the hypothalamic ARC, including neuropeptide Y (NPY), agouti-related peptides (AgRP), proopiomelanocortin (POMC), cocaine and amphetamine regulated transcript (CART), and ghrelin, were measured by Immunofluorescence. The mRNA levels of *UCP-1* in brown adipose tissues and *UCP-2* in white adipose tissues were measured by RT-PCR. The protein levels of UCP-2 in white adipose tissues also were measured by Western Blot. Compared with WT mice, the levels of CART, POMC and ghrelin increased, the levels of NPY and AgRP decreased, whereas the mRNA levels of *UCP-1, UCP-2* increased, the protein levels of UCP-2 increased in *fat-1* mice. The results indicated that the *fat-1* gene or n-3 PUFAs participate in inhibition of body weight by regulating the expressions of appetite neuropeptide and uncoupling protein.

Keywords: Hypothalamus, *fat-1* Gene, Neuropeptide, Body Weight

1. Introduction As the global obesity rate increases, obesity has become a major risk factor for several debilitating and deadly diseases in humans. However, the underlying mechanism of obesity is extremely complex and largely impacted by both environmental and genetic factors[1]. Obesity can be induced by the energy imbalance that is caused by any adverse effect on the regulation of appetite and energy metabolism in the body[2, 3]. The hypothalamus, the control center of ingestive behavior and energy balance in humans, regulates the secretion of various neuropeptides. The hypothalamic arcuate nucleus (ARC), especially in its basal part, is the most important area. ARC plays an important role in appetite regulation and energy balance[4], as two groups of neurons control food intake and energy regulatory systems. The function of feeding inhibition recruits neurons which secrete proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) is to promote the energy metabolism *in vivo* and decrease appetite. On the contrary, the function of feeding stimulation recruits neurons that secrete neuropeptide Y (NPY) and agouti-related peptides (AgRP) is to promote appetite[5]. Ghrelin, a hormone synthesized by a special group of neurons, promotes ingestion, gastrointestinal movement, weight gain, and energy metabolism balance regulation[6, 7]. Uncoupling protein-1 (*UCP-1*) and *UCP-2* play significant roles in energy metabolism because they both increase the energy consumption, especially in adipose tissues[8].

As reported, n-3 PUFAs are critical in regulation of animal body weight and body fat around the abdomen[9, 10, 11]. Generally speaking, mammals are unable to synthesize n-3 PUFAs, and can only take n-3 PUFAs from their diet. The functions of *fat-1* gene are to transform n-6 PUFAs into n-3 PUFAs *in vivo*[12]. Our previous studies showed that taking an n-3 PUFAs-rich diet a few months later, the rats' weight dropped significantly.[13] Moreover, endogenous n-3 PUFAs in *fat-1* mice could reduce adipose tissue weight and control body weight[14, 15]. However, the detailed mechanism that n-3 PUFAs affect body weight is unclear. Here, we investigated how endogenous n-3 PUFAs promote weight loss by regulating the hypothalamic expression of appetite neuropeptide and uncoupling proteins in a *fat-1* transgenic mouse model.

Published at: http://www.ijsciences.com/pub/issue/2019-03/
DOI: 10.18483/ijSci.1960; Online ISSN: 2305-3925; Print ISSN: 2410-4477

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2. Materials and Methods

2.1. Grouping and feeding

Fat-1 transgenic heterozygous mice (a kind gift from Professor Jingxuan Kang， Harvard Medical College and Jianbo Wan，University of Macau) were mated with C57BL/6 Wt mice. To induce the heterologous expression of Caenorhabditis elegans n-3 fatty acid desaturase in mice, Jingxuan Kang et al. modified the *fat-1* gene by optimization of codon usage for mammalian cells and coupled it to a chicken beta-actin promoter (which allows high and broad expression of *fat-1* in mice). Then they microinjected the expression vector into fertilized eggs to produce transgenic mouse lines[12] . Polymerase chain reaction (PCR) was used to identify the offspring of FAT-1 transgenic mice. The male mice were then divided into a *fat-1* transgenic group and a WT group, with an average weight of 21 g. All overweight mice (> 25 g) or underweight mice (< 17 g) were excluded. The six-week-old male heterozygous *fat-1*(+/-) mice and their WT littermates were bred in Qingdao University with access to standard rodent chow as described[15, 16] . They were housed individually in environment-controlled conditions (25±2 ℃, light cycle from 06:00 to 18:00 and dark cycle from 18:00 to 06:00)[17] and allowed *ad libitum* access to food and water throughout the trials. After 8 weeks of cultivation, the animals were killed through cervical dislocation.

2.2． Measurement of body weight and body length

The body weight and body length of each animal were measured weekly. This study was approved and all procedures were performed in accordance with institutional guidelines of the Animal Care and Use Committee of Qingdao University.

2.3. Immunofluorescence

All groups of mice were transcardially perfused with 100 mL of NS, followed by 400 mL 4% (w/v) paraformaldehyde solution in 0.1M phosphate buffer (pH 7.4). The removal brain was fixed in 4% paraformaldehyde for 2 h and then transferred to 30% sucrose solution overnight at 4℃. A series of 14 μm brain coronal sections were cut on a freezing microtome (Kryostat 1720, Leica, Germany)[20] .

Sections were incubated with anti-NPY antibody (polyclonal; dilution, 1:250), anti-AgrP (polyclonal; dilution, 1:500), anti-POMC (polyclonal; dilution, 1:150), anti-CART (polyclonal; dilution, 1:150) and anti-Ghrelin (polyclonal; dilution, 1:500) at 4℃ for 40 h and then incubated with a fluorochrome-labeled secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit IgG; dilution, 1:300) for 2 h. Sections were then mounted with Citifluor. All fluorophores were visualized using a BX50 microscope and images were acquired using a DP50 digital camera (Olympus, Tokyo, Japan). Stained brain sections were imaged using Zeiss LSM 510 laser scanning confocal microscope. All parameters were kept constant for quantification purposes. We used fluorescence intensity and cell counting, and positive cells were used to quantitatively measure cell numbers.

2.4. Real Time PCR

White and brown adipose tissue were homogenized using Trizol reagent (Invitrogen) kit and total RNA was isolated according to the manufacturer's protocol. Total RNA (2 μg) were reversely transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche), and qRT-PCR was performed with Roche LightCycler 480 using SYBR Premix Ex Taq master mix (Roche). The specificity of qRT-PCR was confirmed by agarose gel electrophoresis and melting-curve analysis. A housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase , GAPDH) was used as an internal standard. The primers used are listed in Table 1. The relative fold changes were determined by the method of $2^{-\triangle\triangle Ct}$ as descript[17] . Usually, UCP-1 is mainly secreted by the brown adipose tissues and UCP-2 is mainly secreted by the white adipose tissues，so the mRNA of *UCP-2* was extracted from WAT, the mRNA of *UCP-1* from BAT.

2.5 Western blotting

Anti-UCP-2 and anti-β-actin were purchased from Bioss (China). For western blotting, lysates of white adipose tissue were prepared in RIPA buffer (20 mM Tris, pH 7.5, 5 ml EDTA, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.025% SDS, 1 mM Na-orthovanadate, 10 mM NaF, 25μM β-glycerophosphate) containing protease inhibitors (Roche, Indianapolis, IN). Adipose tissue lysates were prepared from ~200 mg of inguinal WAT or scapular BAT by homogenizing tissues in 1 ml RIPA buffer. After centrifugation at 13,200×g for 15 min at 4℃, the supernatants were subjected to Western Blotting as described[21] . β-actin was used as a loading control.

2.6. Statistical analyses

Data were expressed as mean ± standard deviation (SD). SPSS 18.0 software (SPSS, Inc., Chicago, IL) was utilized to conduct all the statistical analyses of the results. One-way analysis of variance (ANOVA) and Student's t-test were used for the results of the weights of adipose tissues, immunofluorescence and RT-qPCR statistical analysis. Analysis of body weight / height ratio by Two-ways ANOVA. The values were considered statistically significant at $P < 0.05$.

3. Results

3.1. fat-1 gene decreased weight/length ratio.

The *fat-1* transgenic mouse pups at birth were

essentially indistinguishable in size from WT mouse. As shown in Fig.1, the body weight/length ratio of *fat-1* transgenic group was significantly lower than that of WT group. There was a significant difference in body weight/length ratios between *fat-1* transgenic mice and WT mice in the 14th week of age (*fat-1*:2.258 ± 0.02, WT: 2.376 ± 0.04 g/cm, n = 6 animals per group, $P < 0.05$, Fig.1). Two groups of mice eat the same food, each mouse eat 6-8 g per day, no significant difference.

3.2. fat-1 gene changed the levels of appetite neuropeptides.

In order to better understand the causes that contribute to the differences observed in weight/length ratios between the WT and *fat-1* animals, brain sections from both groups were examined by immunofluorescence. The results are shown in Fig.2: the protein expression of three genes, CART, POMC and ghrelin were significantly elevated in *fat-1* transgenic mice relative to their respective WT counterparts. Whereas, NPY, AgRP levels were reduced in *fat-1* transgenic mice.

3.3. Effect of fat-1 gene on mRNA level of UCP-1 and UCP-2

To examine the effect of *fat-1* gene on weight/length ratio between the two groups, RNA extracted from WAT and BAT were examined by RT-PCR. The results are shown in Fig.3: the mRNA expressions of *UCP-1, UCP-2* were significantly elevated in *fat-1* transgenic mice relative to their respective WT controls (Fig3.).

3.4. Effect of fat-1 gene on protein level of UCP-2

Protein extracted from WAT was examined by western blotting. According to Fig.4, the expression level of UCP-2 was significantly elevated in *fat-1* transgenic mice relative to their respective WT controls (Fig4.).

4. Discussion

The hypothalamus is an important center responsible for the synthesis and release of neuropeptides that regulate feeding behaviors and energy balance. Located in the bottom of the third ventricle, ARC, which bulges in the center, is the excitatory and inhibitory neurons that affect appetite. WAT stores excessive energy as triglycerides, while BAT decreases energy storage by generating heat[22] . *UCP-1* and *UCP-2* induce the oxidation and ADP phosphorylation, decrease ATP production, and increase energy consumption and heat yield, eventually leading to weight loss[9, 26, 27] .

Here we found that the *fat-1* gene, which increases the endogenous n-3 PUFA levels by transforming n-6 PUFAs to n-3 PUFAs, obviously reduced weight/length ratios of mice. This result is in accordance with a previous report[17] . The *fat-1* and WT mice showed remarkable differences in the weight/length ratios. Therefore, we speculated that the existence of the *fat-1* gene was responsible for the differences in body weight, length, adipose and hematic parameters. Our data also shows that the expressions of CART, POMC and ghrelin are upregulated, while the expressions of NPY and AgRP are downregulated in the *fat-1* mice compared with the WT mice. The results are in line with our previous study[18]. In the study we measured the relative levels of CART, POMC, NPY, AgRP, ghrelin mRNA in the hypothalamus of *fat-1* transgenic mice and WT mice[18, 19] . Therefore, we think that these expression changes might be a partial mechanism underlying the weight loss in *fat-1* mice. In addition, the increase of *UCP-1* and *UCP-2* is also a reason for body weight reduction in the *fat-1* mice compared with the WT mice (Fig. 4).

The discovery over the last years about the distinct hypothalamic distribution pattern of numerous novel neuropeptides has certainly complicated the hypothalamus[23] . Ghrelin is a newly-found orexigenic neuropeptide that improves appetite. These peptides are the key to better understand how the hypothalamus integrates and coordinates neural and hormonal inputs/signals into neuroendocrine, autonomic and behavioral responses. Generally, the appetite of animals can be promoted by the elevation in NPY and AgRP levels, but can be reduced by the increase in the POMC and CART levels[24] . Interestingly, ghrelin is highly expressed in the *fat-1* mice, which is contrary to the reduction of body weight. Ghrelin is considered as a compensatory mechanism that actively protects metabolic balance[25]. The function of ghrelin could be decreased by the reduction in NPY and AgRP levels[8, 19] . Here, we speculate that the high levels of ghrelin neuropeptides in *fat-1* mice are needed to maintain normal action and compensate for the low levels of NPY and AgRP. Nevertheless, the mRNA levels of *UCP-1* and *UCP-2* are upregulated in *fat-1* mice, which might be responsible for the body weight reduction.

In conclusion, *fat-1* gene could reduce body weight by modulating the hypothalamic expressions of appetite neuropeptides and UCP proteins in adipose. Nonetheless, to understand obesity and develop novel therapies, we need further studies to determine the precise molecular mechanisms how the *fat-1* gene regulates body weight.

Acknowledgements

This work was supported by the National Natural Science Foundations of China (No. 30772000); Natural Science Foundations of Shandong Province (No.ZR2018MH009).

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Figure Legends

Fig.1. Body Weight/Length ratios of Fat-1 transgenic mice and wild-type mice. The body weight/length ratio of *fat-1* group was significantly lower than that of WT group. Values are expressed as means ± SEM (t=10.40, *P*<0.05). *fat-1*, Fat-1 transgenic group and WT, wild-type group. a means *P*<0.05 vs. WT. n=6 animals per group.

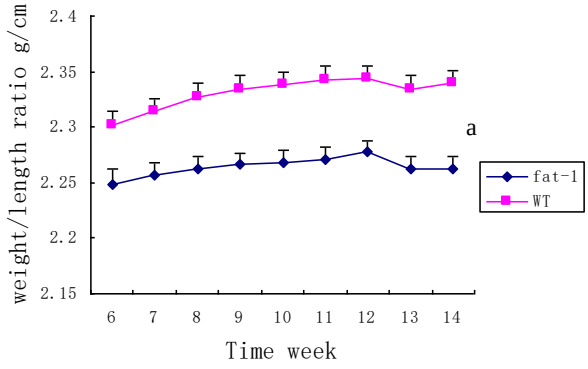
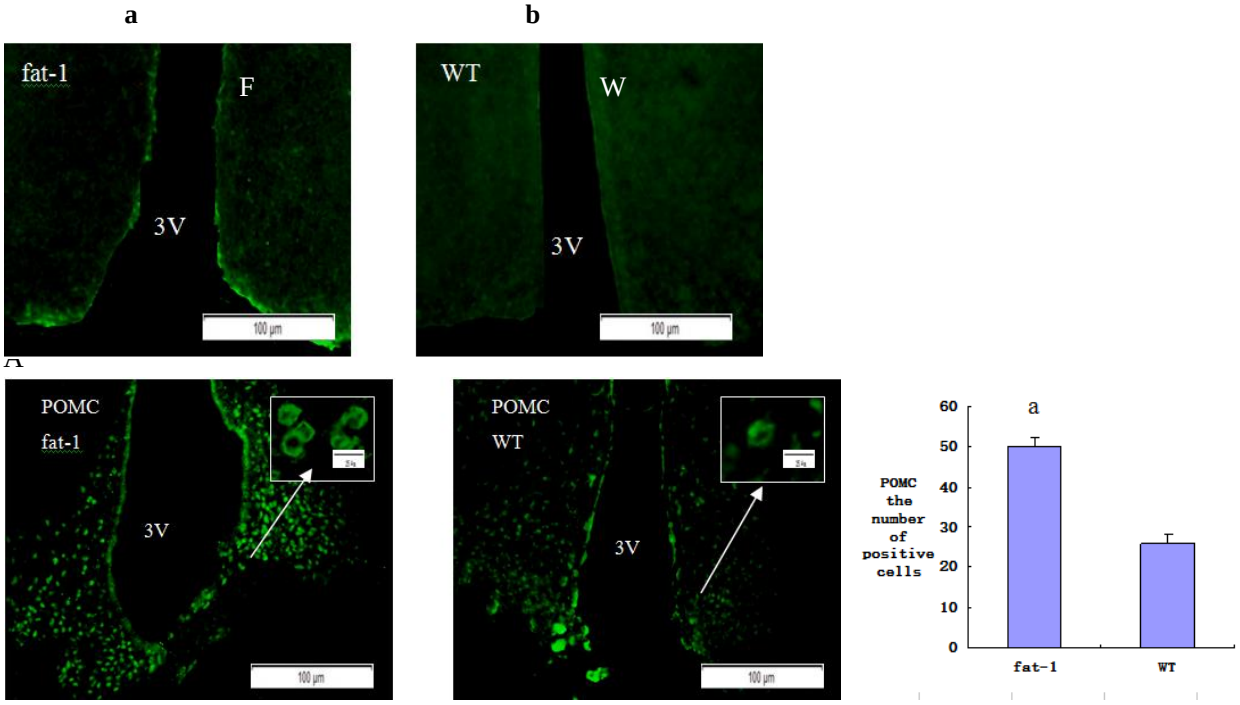
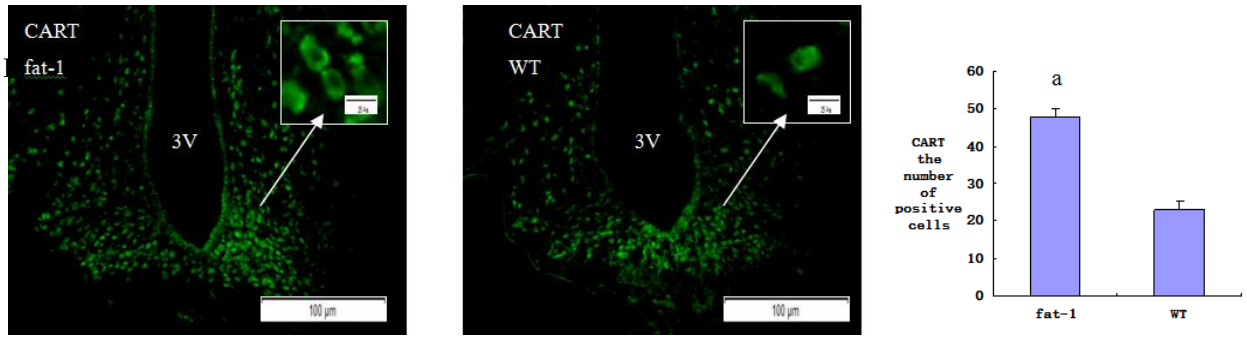


Fig.2. Representative immunofluorescence images highlights the expression of POMC, CART, NPY, AgRP and Ghrelin in neurons belonging to the hypothalamic arcuate nucleus. POMC (a), CART (b) and ghrelin were significantly elevated in *fat-1* mice than that of WT mice. On the contrary, NPY and AgRP levels were reduced in *fat-1* mice. The negative control showed background staining (F, W). fat-1：Fat-1 transgenic group，WT：wild-type group. The statistical chart is used to count the positive cells quantitatively. Scale bar is 100μm. Scale bar of the squares inset the images is 25μm. Bar graphs compare POMC (A), CART (B), NPY (C), AgRP (D), and ghrelin (E) levels in *fat-1* and WT mice. Values are expressed as means ± SEM. fat-1：Fat-1 transgenic group，WT：wild-type group. a means P < 0.05 vs. WT, n = 6 animals per group.




CART
fat-1
3V
100 μm
CART
WT
3V
100 μm
a
CART the number of positive cells
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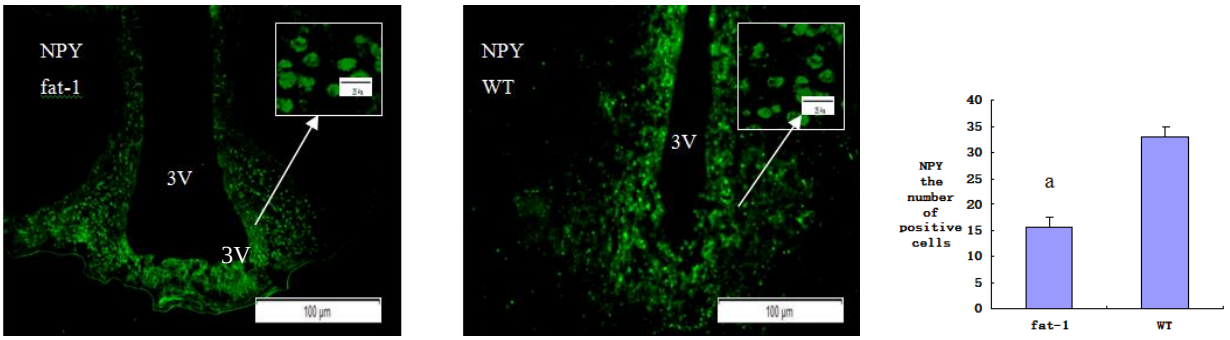
50
40
30
20
10
0
fat-1
WT

C


NPY
fat-1
3V
3V
100 μm
NPY
WT
3V
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a
NPY the number of positive cells
40
35
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25
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15
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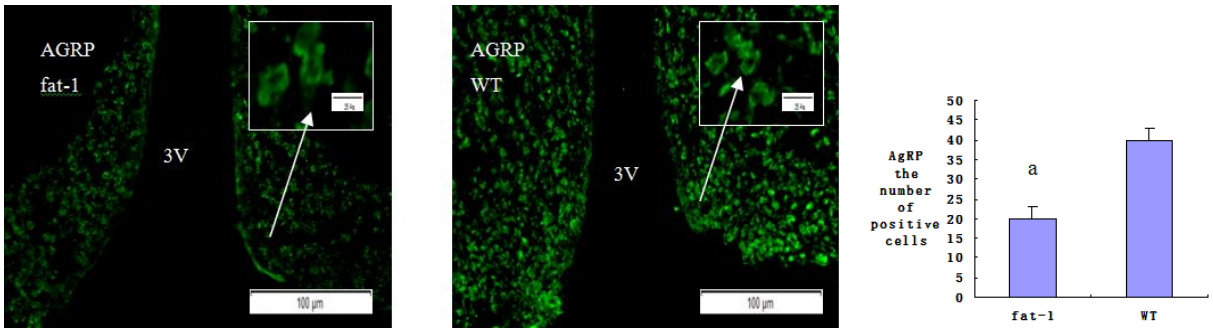
5
0
fat-1
WT

D


AGRP
fat-1
3V
100 μm
AGRP
WT
3V
100 μm
a
AgRP the number of positive cells
50
45
40
35
30
25
20
15
10
5
0
fat-1
WT

E

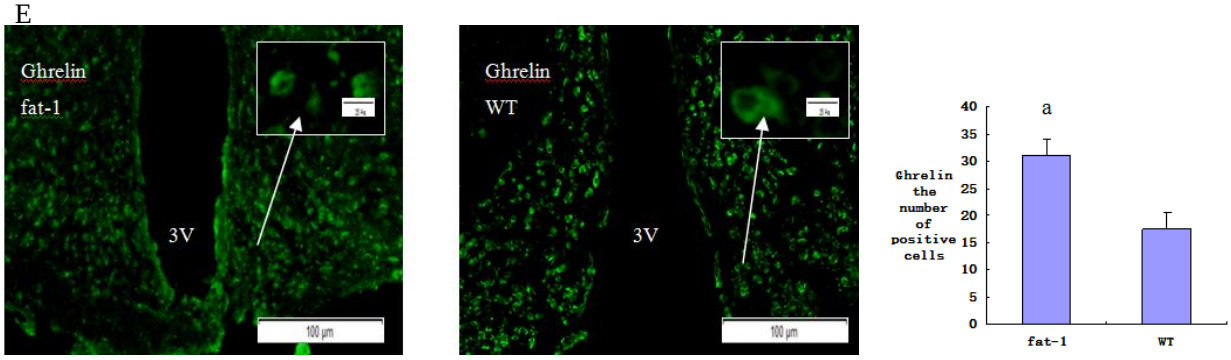

Ghrelin
fat-1
3V
100 μm
Ghrelin
WT
3V
100 μm
a
Ghrelin the number of positive cells
40
35
30
25
20
15
10
5
0
fat-1
WT

Fig.3. Comparisons of *UCP-1* (A) and *UCP-2* (B) mRNA in *fat-1* transgenic mice and wild-type mice. The mRNA expressions of *UCP-1* (A) and *UCP-2* (B) were significantly elevated in *fat-1* mice compared to WT mice. Values are expressed as means ± SEM. fat-1：Fat-1 transgenic group，WT：wild-type group. a means P<0.05 vs. WT. n=6 animals per group.

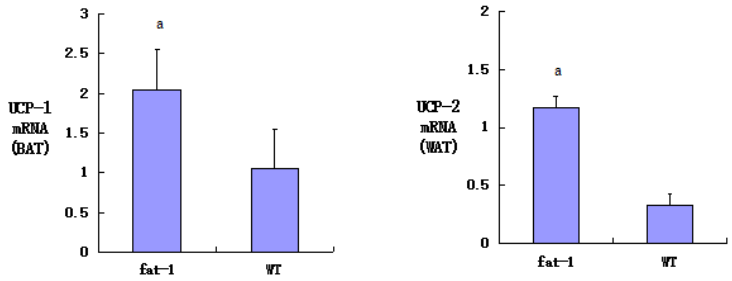


Fig.4. Comparisons of UCP-2 protein in fat-1 transgenic mice and wild-type mice. The protein expressions of UCP-2 were significantly elevated in fat-1 mice compared to WT mice. Values are expressed as means ± SEM. fat-1：Fat-1 transgenic group，WT：wild-type group. a means P<0.05 vs. WT. n=6 animals per group.