Life Sciences 226 (2019) 12–21

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Regulatory role of metallothionein-1/2 on development of sex differences in a high-fat diet-induced obesity

Takashige Kawakami*, Satoshi Takasaki, Yoshito Kadota, Daiki Fukuoka, Masao Sato, Shinya Suzuki

Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan

ARTICLE INFO

Keywords:
Adipose tissue
Metallothionein
Obesity
Androgen
Estrogen
Sex differences

ABSTRACT

Aims: To evaluate the role of metallothionein (MT) in sex differences of obesity, we examined the effect of MT on regulation of lipid accumulation in female and male wild type (WT) and MT1/MT2-null (MT-KO) mice.
Main methods: Male and female WT and MT-KO mice fed standard diet (SD) or high-fat diet (HFD) for 35 weeks. Surgical castration in male mice was also performed to examine the effects of androgen on fat accumulation under HFD condition.
Key findings: The fat mass and size of adipocytes in white adipose tissue (WAT) was greater in adult MT-KO mice than in WT mice after 35 weeks of SD feeding without gender differences, suggesting a role of MT in limiting WAT development during normal growth in both sexes. In female mice fed HFD, weights of WAT and body were greater in MT-KO mice than in WT mice, indicating that MT had a preventive role against excess fat accumulation. In male mice fed HFD, WAT weight hardly increased in MT-KO mice compared to the increase in WT mice. Surgically castrated WT males fed HFD had lower WAT weight compared with sham-treated mice, although castrated MT-KO males fed HFD had greater increases in WAT weight compared with sham-treated mice and castrated WT males.
Significance: These data suggest that MT could enhance the preventive action of estrogen against excess fat accumulation, on the contrary, MT augmented the ability of androgen to increase fat accumulation. MT may act to modify the susceptibility to obesity under sex hormones.

1. Introduction

Excessive fat and energy intake can lead to the development of obesity, which induces obesity-related diseases including type-2 diabetes, cardiovascular disease, and cancer [1]. To reduce the incidence of such diseases, it is important to decrease excessive visceral obesity. Obesity is characterized by an increase in adipose tissue mass with the production and/or deposition of new adipocytes. Effective diagnosis and treatment require the identification of regulatory factors involved in fat accumulation, especially those that can prevent obesity. Kenyon emphasized that adipose tissue plays a central role in longevity, and interventions restricted to adipose tissue may impact life span [2]. The importance of adipose tissue is underlined by the observation that mice lacking the insulin receptor in adipose tissue are long-lived [3]. Conversely, obesity may represent a state of accelerating aging [4].

Metallothionein (MT) is a low-molecular-weight and cysteine-rich protein that plays a regulatory role in the metabolism of essential metals such as zinc and copper [5,6]. MT is induced by various stimuli, including metals, reactive oxygen species (ROS), inflammatory cytokines, and hormones [7]. These proteins also play preventive roles against damage associated with heavy metal toxicity caused by cadmium and mercury, oxidative stress [8], and endoplasmic reticulum (ER) stress [9].

It is reported that MT has a potential to prevent obesity and obesity-related diseases [10,11]. In a previous study, we demonstrated that female MT1/MT2-null (MT-KO) mice were shown to develop high-fat diet (HFD)-induced obesity compared to wild-type (WT) mice, while standard diet (SD) did not induce obesity in these mice [12]. Although there is a sex difference in obesity in mammals [13,14], the mechanism remains poorly understood because of multiple effects of sex hormones on fat metabolism. To better understand the relationship between the role of MT and obesity, it becomes necessary to study whether MT is related to sex differences in fat accumulation in adipose tissue. In addition, we observed that MT-KO mice of both sexes had shorter life spans than WT mice [15]. As mentioned above, adipose tissue is significant not only in the development of obesity-related diseases, but

* Corresponding author.
E-mail address: tkawakami@ph.bunri-u.ac.jp (T. Kawakami).

https://doi.org/10.1016/j.lfs.2019.04.012
Received 13 February 2019; Received in revised form 1 April 2019; Accepted 3 April 2019
Available online 04 April 2019

Table 1
Primer sets used for RT-PCR analysis. PCR was performed using the primer sets indicated as below under optimal amplification (95 °C for 5 min; 24–33 cycles of 95 °C for 30 s, 58–60 °C for 30 s, 72 °C for 45 s; 72 °C for 5 min) for each gene.

Gene	Primer sequence	
	Forward	Reverse
36B4	5′-GAGATTCGGGATATGCTGTTGG-3′	5′-GTTGTCAAACACCTGCTGGATG-3′
Adipoq	5′-CAGGATGCTACTGTTGCAAGC-3′	5′-TGCAGTCAGTTGGTATCATGG-3′
Cebpa	5′-AAGCCAAGAAGTCGGTGGA-3′	5′-CAGTTCACGGCTCAGCTGTT-3′
Mest	5′-AACCGCAGAATCAACCTGCT-3′	5′-CGAAGAAATTCATGAGCCTGG-3′
Pparγ	5′-GGTGAAACTCTGGGAGATTC-3′	5′-TAATAAGGTGGAGATGCAGG-3′
Retn	5′-CCACGTACCCACGGGATGAA-3′	5′-TCAGGAAGCGACCTGCAGCTTACA-3′
Serpine1	5′-GTTCACTTTACCCCTCCGAGAA-3′	5′-CAAAGATGGCATCCGCAGT-3′
Vldlr	5′-TCAGAAGCTGTTTTGGGCTG-3′	5′-GCATGTGCAACTTGGAATCC-3′

also in life span. It also may be important to study the role of MT in adipose tissue for elucidation of the effect of MT on life span extension.

In the present study, effects of SD and HFD on fat accumulation in MT-KO and WT mice were examined in both males and females. We focused on three phenomena: MT-mediated regulation of fat content in normal adipose tissue; MT-mediated modulation of fat accumulation in adipose tissue induced via HFD; and expression of genes related to lipid accumulation to clarify the factors contributing to obesity.

2. Materials and methods

2.1. Animals

MT-KO mice (129/Sv-MT1MT2$^{\mathrm{tml\ bri}}$) developed by Masters et al. [16] and 129/SvCPJ (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Only mice of the 129/SvCPJ strain were used for mating to maintain the same genetic background. The mice were housed in a controlled room (temperature, 22 °C; humidity 55 ± 5%) under 12-h light/dark cycles, fed chow diet (MF, Oriental Yeast Co. LTD., Tokyo, Japan), and watered ad libitum. Laboratory chow diet (CE-2, CLEA Japan, Osaka, Japan) containing 4.4% oil (344 kcal/100 g) was administered as the SD. The HFD (CLEA Japan) contained 15.88% beef tallow, 20% safflower oil, 24.5% milk casein, 5.0% ovoprotein, 0.43% L-cysteine, 5.5% cellulose powder, 6.74% sucrose, 6.925% lactose, 1.4% vitamin mix (AIN-93), 5.0% mineral mix (AIN-93G-MX), 0.4%, 8.25% maltodextrin, and 0.0002% butylhydroquinone. WT and MT-KO mice were fed the SD or HFD beginning at eight weeks of age for 35 weeks. The SD and HFD foods were changed and weighed once a week. Food intake was calculated by subtracting the weight of the remaining food from the weight of the total food supplied during that week. Calorie intake was calculated based on 340 kcal/100 g for the SD and 507.6 kcal/g for the HFD. Caloric values were obtained from CLEA Japan, Inc. The HFD experiment was conducted at two independent trials using identical condition. For the surgical castration experiment, WT and MT-KO mice aged 6–7 weeks were anesthetized with pentobarbital combined with isoflurane, and castration was performed as described [17]. All experimental procedures were approved by the Animal Care and Use Committee of Tokushima Bunri University (Approval number 67) and conformed to the guidelines established by the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

2.2. Measurements

The body weight of each animal was measured weekly for 35 weeks. Then, the animals were dissected under isoflurane, and each organ was removed. White adipose tissue (WAT) surrounding the ovaries, testes, kidneys, and posterior belly film was weighed. The dorsoventral subcutaneous fat pads of seven-day-old male and female mice were also weighed. WAT samples were fixed in 10% neutral-buffered formalin and processed for paraffin embedding. They were then sectioned and stained with hematoxylin–eosin. The adipocyte sizes in WAT sections from different mice were measured using Motic Images Plus 2.0S software (Shimazu Rika, Tokyo, Japan). Before blood sampling, the mice were fasted for 6 h to reduce the effects of dietary intake on plasma constituents. Plasma leptin levels were measured with a mouse leptin kit (Morinaga Institute of Biological Science, Tokyo). Plasma estradiol and testosterone levels were measured with estrogen and testosterone kits (Cayman Chemical Co., Ann Arbor, MI, USA), respectively.

2.3. RNA isolation and RT-PCR

Frozen tissues were homogenized in TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) using a Polytron homogenizer. Total RNA was extracted from WAT of WT and MT-KO mice using an RNeasy® Mini Kit (QIAGEN, Germantown, MD, USA). Total RNA samples (2 μg) were reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per the manufacturer's instructions, followed by reverse transcription polymerase chain reaction (RT-PCR) analysis, which was previously described [18]. The primer sequences are shown in Table 1. The PCR products were resolved on 2% agarose gels and stained with ethidium bromide.

2.4. Statistical analysis

The data were represented as mean ± S.E.M values. The mean significant difference was determined using one-way ANOVA, followed by the Tukey test or Student *t*-test as applicable. The threshold of statistical significance was set at $p < 0.05$.

3. Results

3.1. Body weight change in male and female WT and MT-KO mice with SD or HFD feeding for 35 weeks

WT- and MT-KO mice of either gender were eight weeks old at the beginning of the experiments and showed no gross phenotypic differences. After 35 weeks, female HFD-fed MT-KO mice had a greater body weight compared with SD-fed MT-KO mice, whereas HFD-fed female WT mice had slightly greater body weight compared to SD-fed WT mice (Fig. 1a, right).

In males, the body weight was greater in SD-fed MT-KO mice than in SD-fed WT mice (Fig. 1a, left). Our observation is similar to previous report that male MT-KO mice, which have mixed 129/Ola and C57BL/6J genetic background, had increased body weight compared with C57BL/6J mice under SD condition [10]. In male MT-KO mice, a HFD had the similar effect as a SD on body weight (Fig. 1a, left). However, male HFD-fed WT mice showed greater body weight compared with SD-fed WT mice (Fig. 1a, left). Amounts of calorie intake in mice fed HFD were greater than those in mice fed SD, and differences were not observed between WT and MT-KO mice for three months in each sex (Fig. 1f).

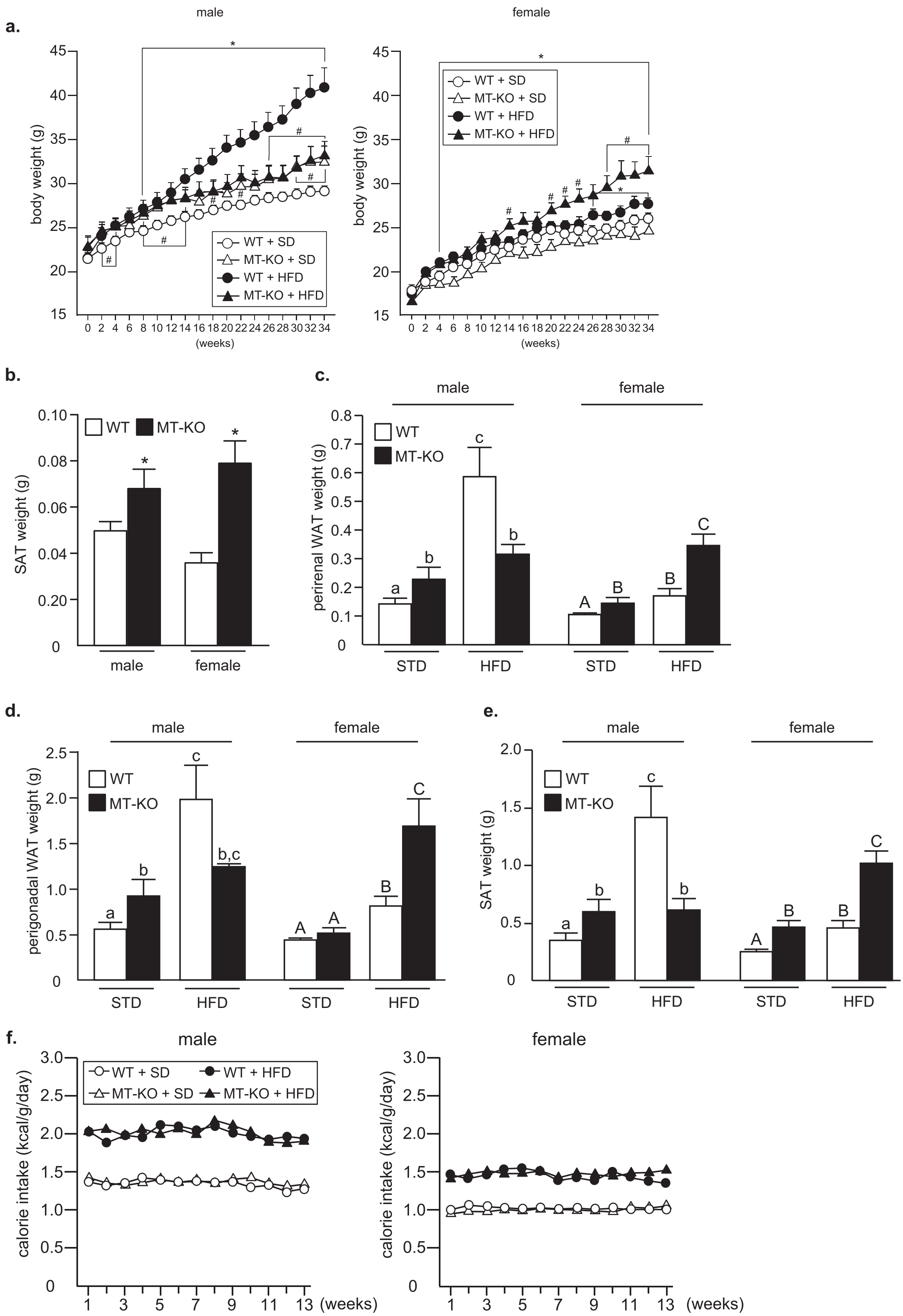

Fig. 1. Growth of WT and MT-KO mice fed SD or HFD for 35 weeks.
(a) Body weight of male and female mice. Mice were weighed every week. Values are means ± SEM for groups of 7–10 mice. $^*p < 0.05$ compared to mice fed with SD. $^\#p < 0.05$ compared to WT mice. Changes in (b) subcutaneous adipose tissue (SAT) at seven days after birth in male and female wild-type (WT) and MT-knockout (MT-KO) mice; (c) perirenal WAT, (d) perigonadal WAT, and (e) SAT of male and female mice fed SD or HFD for 35 weeks. Male (left) and female (right) mice, and WT (open column) and MT-KO (closed column) mice. (f) Calorie intake in the following weeks for the mice fed with SD or HFD in male and female WT and MT-KO mice. Student's *t*-test was used for statistical analysis (panels a and b). Values are means ± SEM for groups of seven to 10 mice. $^*p < 0.05$ compared to mice fed with SD. Different letters signify statistical significance ($p < 0.05$) using Tukey and one-way ANOVA tests (panels c to e). Statistical results are indicated by lower-case character(s) among males and upper-case character(s) among females.

3.2. Greater fat mass in MT-KO mice compared with WT mice in neonatal period and SD condition for 35 weeks in both sexes

Next, we compared the weight of adipose tissues in SD-fed WT and MT-KO mice in the neonatal period. In both males and females at seven days after birth, the weight of subcutaneous adipose tissue (SAT) was greater in MT-KO mice than in WT mice (Fig. 1b). There was little perirenal or perigonadal WAT in seven-day-old mice. After 35 weeks in SD condition, the weight of perirenal and perigonadal WAT (Fig. 1c and d) and SAT (Fig. 1e) was significantly greater in MT-KO mice than in WT mice in males. In female SD-fed mice, weight of the perirenal WAT (Fig. 1c) and SAT (Fig. 1e) was greater in MT-KO mice than in WT mice. These data showed that MT defect resulted in greater fat mass of WAT and SAT in SD-fed mice compared with that in the corresponding WT mice, indicating that MT had a role in limiting adipose tissue development.

3.3. Fat mass and adipocyte size markedly increased in male WT and female MT-KO mice after HFD feeding for 35 weeks

Although the rate of weight gain was different for adipose tissue after SD or HFD feeding for 35 weeks, the weight of all adipose tissue (Fig. 1c–e) was well correlated with body weight (Fig. 1a). In females, the weight of perirenal and perigonadal WAT and SAT was greater in HFD-fed MT-KO mice than in SD- or HFD-fed WT mice (Fig. 1c–e). Conversely, in males, the weight of perirenal and perigonadal WAT and SAT was greater in HFD-fed WT mice than in SD- or HFD-fed MTKO mice (Fig. 1c–e).

HFD feeding-induced adipocyte enlargement appeared prominently in male WT mice and female MT-KO mice (Fig. 2a). The size/number distribution of adipocytes in WAT showed that the number of adipocytes larger than 3000 μm^2 was greatly increased by HFD in female MT-KO mice and male WT mice (Fig. 2b). In the SD-fed group, cell sizes were slightly but significantly greater in MT-KO mice than in WT mice in each sex (Fig. 2a, b, and c, left). These data suggested that MT had a possible role in maintaining adipocyte cell size during SD feeding in both sexes. Cell size in HFD-fed mice (Fig. 2a, b, and c, right) increased more than in SD-fed mice in both males and females. In HFD-fed female mice, cell size was significantly greater in MT-KO mice than in WT mice (Fig. 2c). Therefore, MT had a role in maintaining proper adipocyte size, even during excess fat feeding. Conversely, in male HFD-fed mice, cell size was significantly greater in WT mice than in MT-KO mice (Fig. 2c).

Next, we analyzed the mRNA expression levels of adipocyte differentiation-related and enlargement-related genes in adipose tissue of WT and MT-KO mice. The mRNA levels of mesoderm-specific transcript (Mest) (Fig. 3a) and very low-density lipoprotein receptor (Vldlr) (Fig. 3b) were approximately 1.5–1.7-fold greater in MT-KO mice than in WT mice in males and females after SD feeding for 35 weeks. HFD feeding for 35 weeks further increased the mRNA expression of Mest (Fig. 3a right) and Vldlr (Fig. 3b right) in both male and female mice, and the rate of increase of these mRNA levels in males that were fed HFD was greater in WT mice than in MT-KO mice. Conversely, in females, the mRNA increase rate caused by HFD feeding was greater in MT-KO mice than in WT mice. The mRNA levels of Mest (Fig. 3a) and Vldlr (Fig. 3b) were well-correlated with adipocyte size in WATs of male and female mice (Fig. 3c and d).

Since leptin is a key molecule involved in the regulation of energy balance, we investigated the level of leptin in serum and mRNA expression level of leptin in WAT. No significant differences were

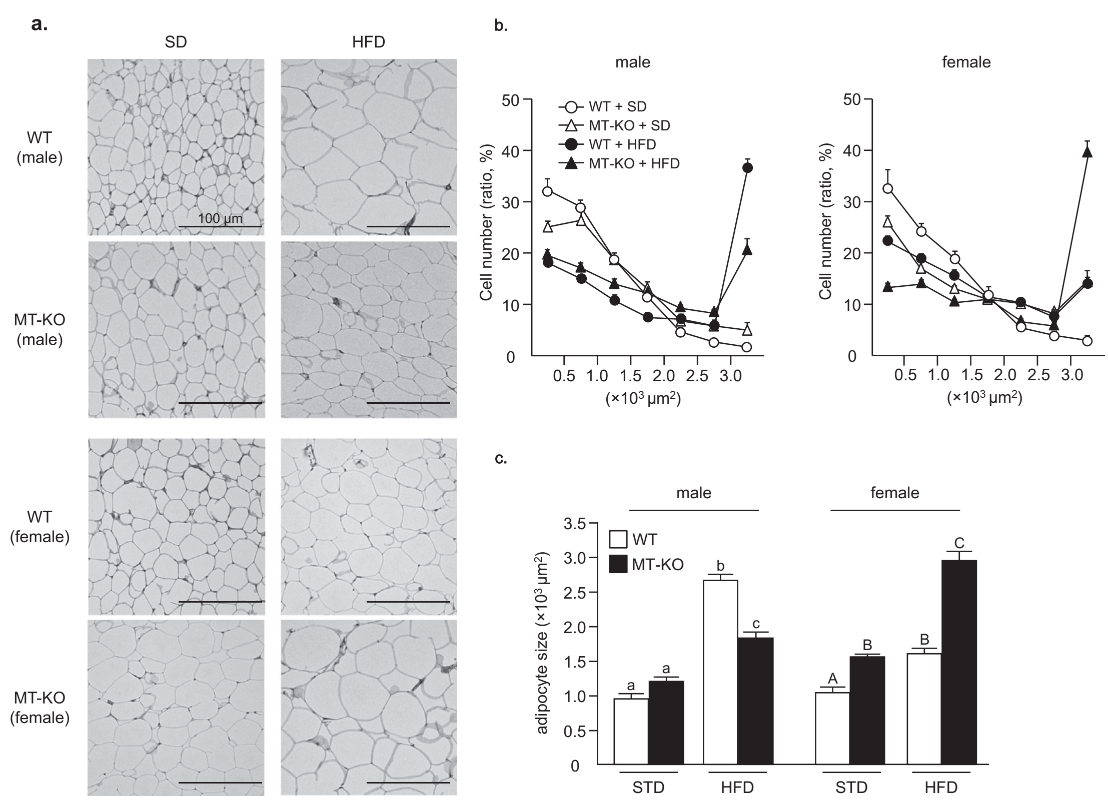

Fig. 2. Effects of 35-week HFD feeding on the number and size of adipocytes in male and female MT-KO and WT mice.
(a) Histological analysis of visceral WAT (3-μm-thick sections stained with hematoxylin and eosin). Typical microscopic images from five independent mice per group are shown; scale bar = 100 μm. Mice fed SD (left), and mice fed HFD for 35 weeks (right); (b) adipose cell-size distribution rate; (c) adipocyte size in visceral WAT. The sizes of adipocytes in five WAT sections from independent mice were measured; therefore, 500 cells were measured per group. Values are mean ± S.E.M. of eight to 10 mice per group. Male (left) and female (right) mice. Different letters signify statistical significance ($p < 0.05$) using Tukey and one-way ANOVA tests. Statistical results are indicated by lower-case character(s) among males and upper-case character(s) among females.

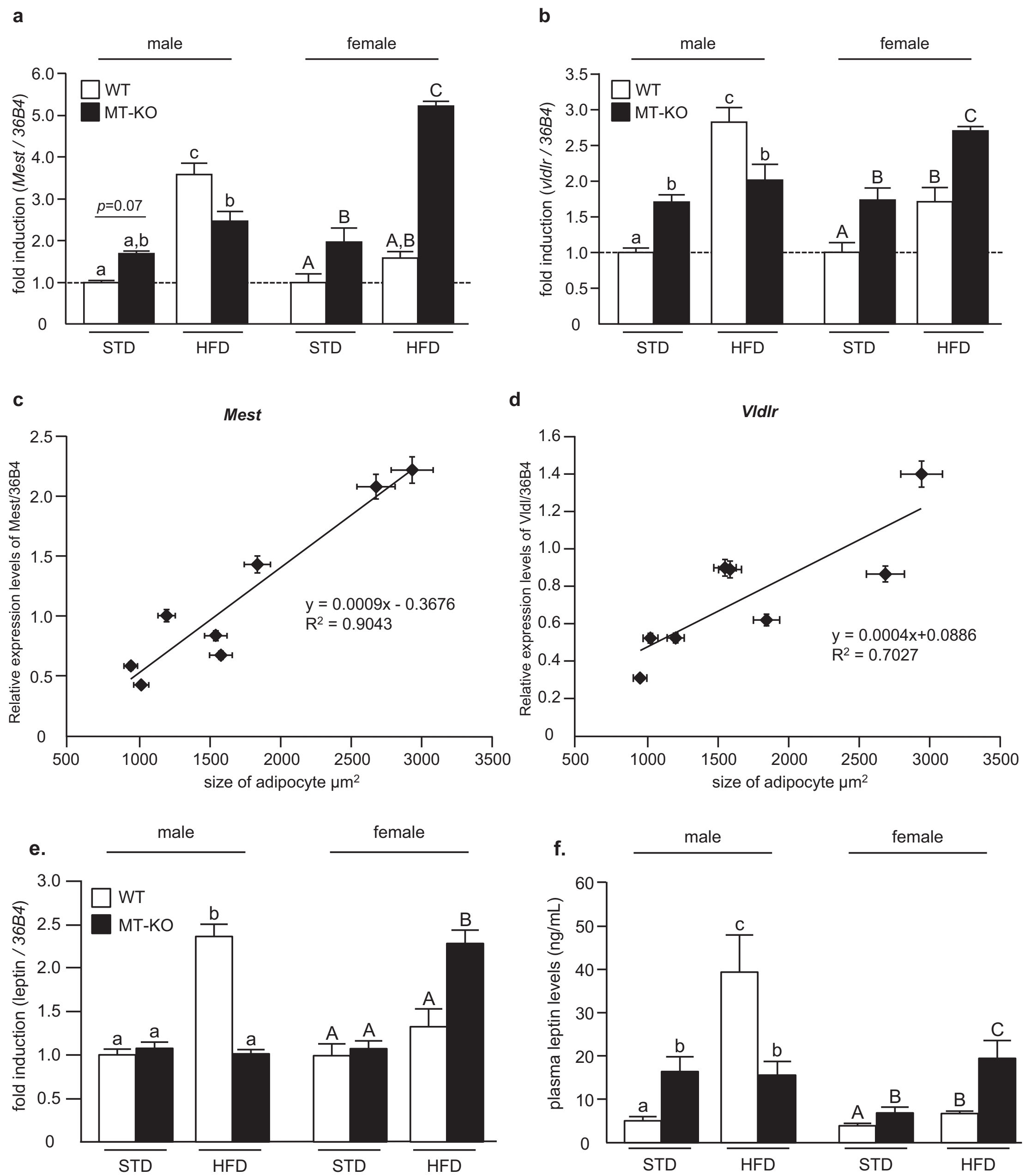

Fig. 3. Effects of 35-week HFD feeding on the mRNA levels of adipocyte differentiation and adipocyte enlargement-related genes in male and female WT and MT-KO mice.

(a) Relative mRNA expression levels of Mest and (b) Vldlr. Correlation of expression of (c) Mest and (d) Vldlr with adipocyte size in WATs of male and female mice. (e) Relative mRNA expression levels of Lep, and (f) plasma leptin level. Values are expression levels in SD-fed WT mice, as quantified via densitometry and normalized to 36B4 mRNA levels in four mice per group. Values are means ± S.E.M. of four mice per group. Different letters signify statistical significance ($p < 0.05$) using Tukey and one-way ANOVA tests. Statistical results are indicated by lower-case character(s) among males and upper-case character(s) among females.

observed in basal levels of leptin mRNA expression in both sexes of SD-fed WT and MT-KO mice (Fig. 3e). Plasma leptin levels were greater in MT-KO mice than in WT mice in each sex after 35 weeks of SD feeding. HFD feeding increased plasma leptin and mRNA expression of leptin in WAT in male WT and female MT-KO mice (Fig. 3e and f).

The mRNA expression of factors responsible for adipocyte differentiation such as peroxisome proliferator-activated receptor γ (Pparγ) and members of the CCAAT enhancer binding proteins (CEBPs) family such as Cebpα, Cebpβ, and Cebpδ depended neither on HFD feeding nor MT-KO in each sex (Fig. 4). Expression of fatty acid binding protein 4 (Fabp4), which was responsible for lipid transport, was increased by HFD feeding, although there were no differences in Fabp4 expression between WT and MT-KO mice (Fig. 4).

3.4. Changes in the level of adipokines and metabolic factors involved in obesity-related diseases

The expression of Serpine1 (Serpin Family E Member 1), which encoded plasminogen activator inhibitor-1 (PAI-1) in WAT of SD-fed mice was much greater in MT-KO mice than in WT mice in each sex (Fig. 5a). HFD feeding further increased Serpine1 expression in male and female WT and MT-KO mice to a similar extent. These data showed that Serpine1 expression was induced in mouse WAT by MT-KO and HFD feeding regardless of sex. SD-fed WT and MT-KO mice did not

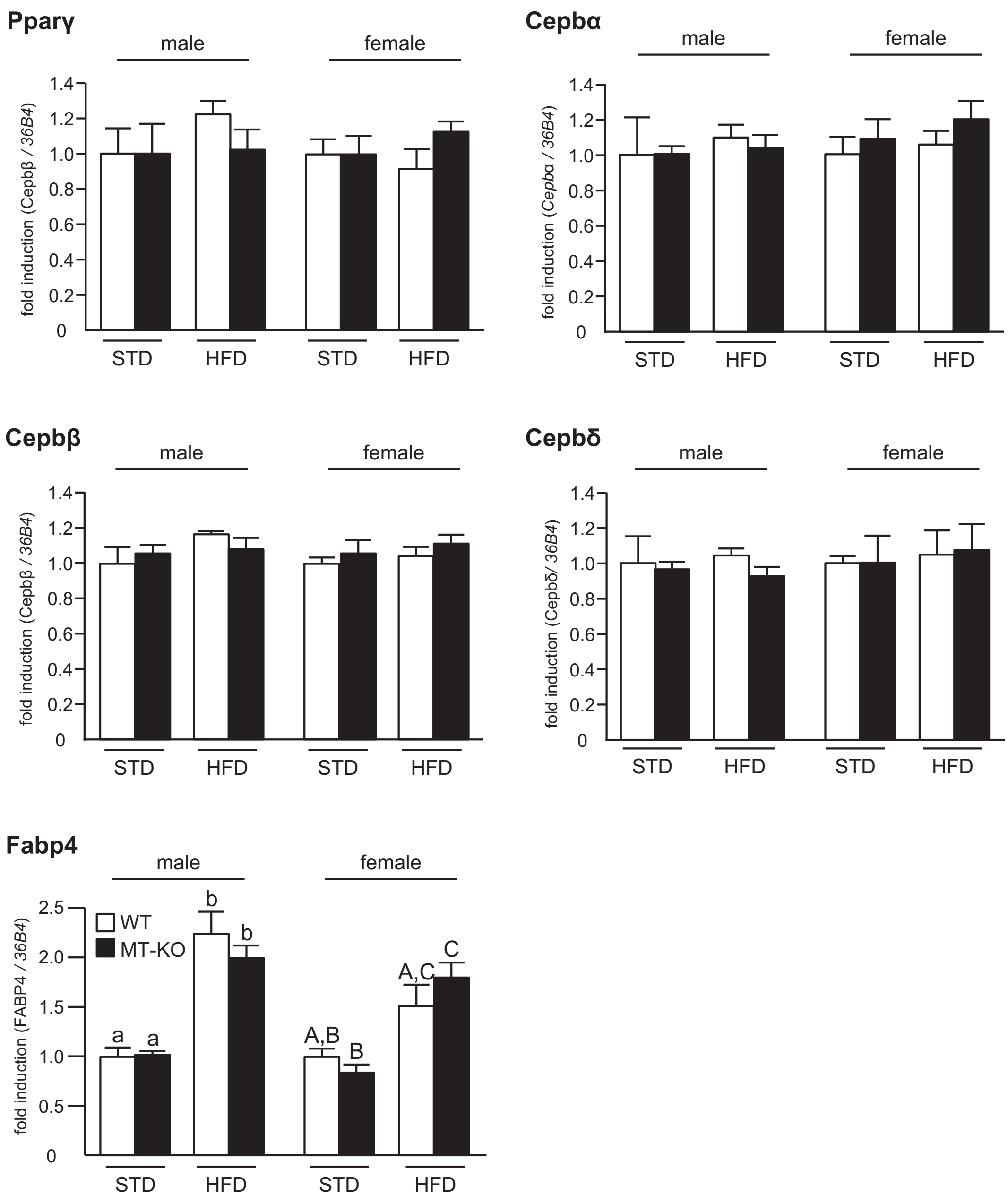

Fig. 4. Effects of 35-week HFD feeding on the mRNA levels of adipocyte differentiation-related genes in male and female WT and MT-KO mice.
Relative mRNA levels of Pparγ, Cebpα, Cebpβ, Cebpδ, and Fabp4 in WATs of male and female mice. Values are expression levels in SD-fed WT mice, as quantified via densitometry and normalized to 36B4 mRNA levels in four mice per group. Values are mean ± SEM for groups of four mice. Different letters signify statistical significance ($p < 0.05$) using Tukey and one-way ANOVA tests. Statistical results are indicated by lower-case character(s) among males and upper-case character(s) among females.

show significant differences in Adipoq (Adiponectin, C1Q And Collagen Domain Containing) mRNA levels in WAT, and HFD feeding also did not result in any changes in the levels (Fig. 5b). HFD feeding increased the expression of Resistin (Retn) in WAT of all mice (Fig. 5c).

3.5. Changes in plasma concentration of sex hormones

Sex differences in sensitivity to HFD feeding between WT and MT-KO mice were observed in body weight gain and changes in the weight, adipocyte size, and mRNA expressions associated with lipid accumulation of mouse WAT. In order to investigate whether these sex differences were caused by sex hormone levels in mouse blood, we measured plasma concentrations of estradiol and testosterone as female sex hormone and male sex hormone, respectively. There were no significant differences in plasma concentration of estradiol and testosterone between WT and MT-KO mice, and HFD feeding for 35 weeks did not influence the sex hormone levels (Fig. 6).

3.6. Effect of surgical castration on total body weight and WAT weight in HFD-fed WT and MT-KO mice

Androgen-associated changes in body and WAT weight are known to impact the obesity process, but the interaction with MT remains unclear. Therefore, we investigated the effect of surgical castration on total body weight and WAT weight in HFD-fed WT and MT-KO mice. In WT mice, surgical castration treatment slightly reduced body weight gain, especially from week 6 until week 10, but there was not statically significant difference between sham-operated and castrated mice

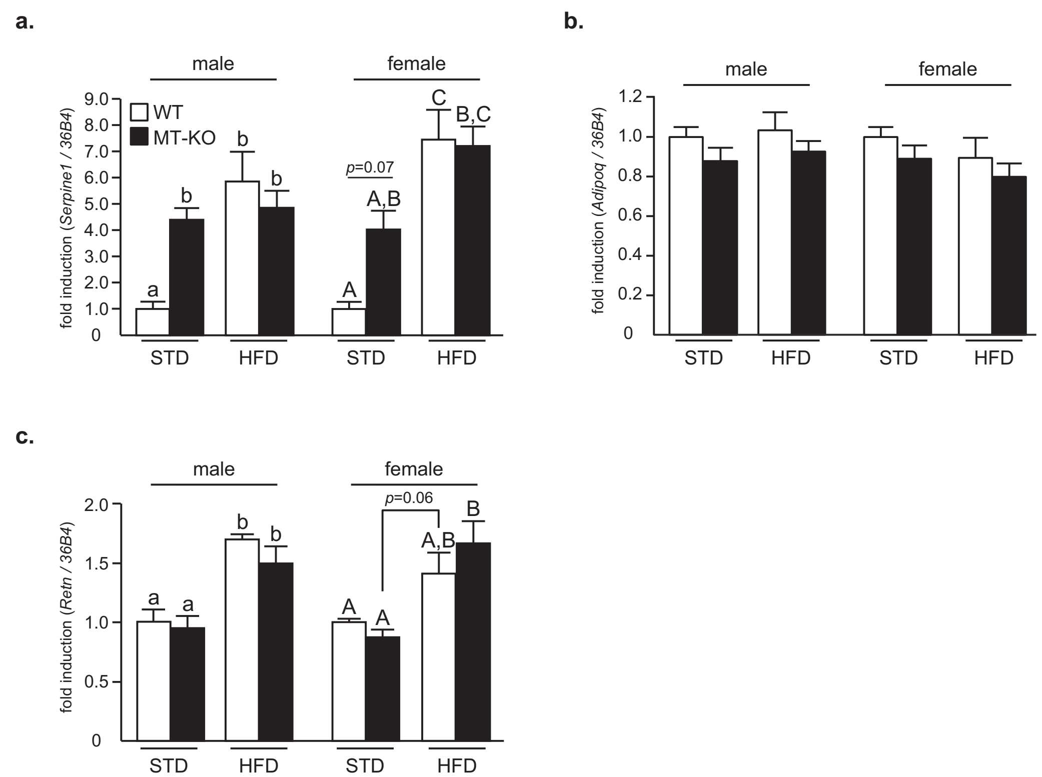

Fig. 5. Effects of 35-week HFD feeding on mRNA levels of adipocytokines in male and female WT and MT-KO mice. The mRNA levels of (a) *Serpine1*, (b) *Adipoq*, (c) *Retn* in WAT of male and female WT and MT-KO mice. The mRNA levels of the genes in WAT specimens were normalized as a ratio to the corresponding 36B4 mRNA expression level. Values are mean ± S.E.M. of four mice per group. Different letters signify statistical significance ($p < 0.05$) using Tukey and one-way ANOVA tests. Statistical results are indicated by lower-case character(s) among males and upper-case character(s) among females.

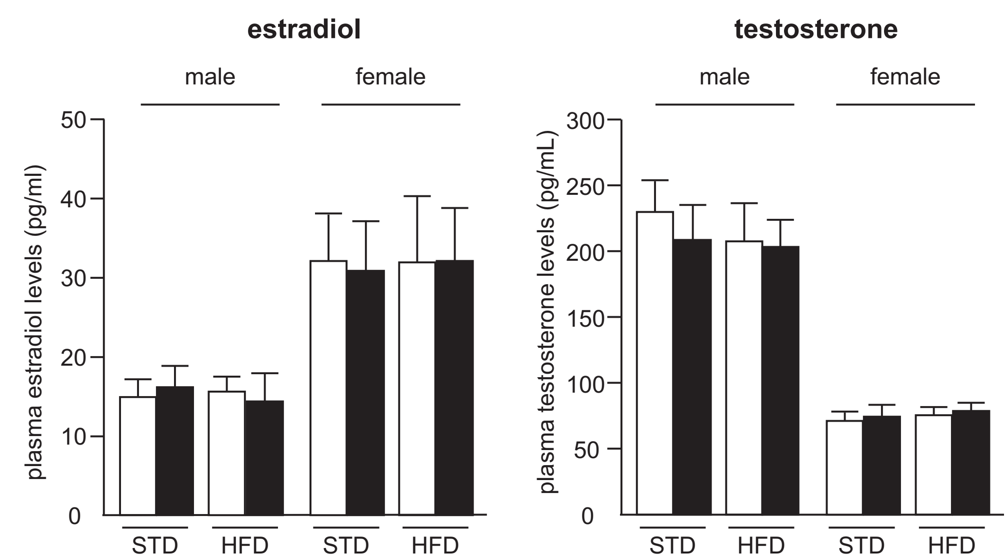

Fig. 6. Effects of 35-week HFD feeding on the serum estradiol, and testosterone levels in male and female WT and MT-KO mice. Plasma estradiol and testosterone levels. Data are expressed as mean ± SEM in groups of four mice.

(Fig. 7a, left). The data suggest that androgen does not affect HFD-induce body weight gain in the present of MT. However, depletion of androgen by castration significantly suppressed fat accumulation induced by HFD in WT mice (Fig. 7b). On the contrary, body weight gain and fat accumulation were greatly increased in castrated MT-KO mice fed HFD (Fig. 7a, right, and b). These data suggested that the conflicting action of androgen against WAT was dependent on the presence of MT.

4. Discussion

MT mRNA has been detected in human SATs [19], and it has been shown that fat cells in WAT secrete MT [20]. The present study showed that seven-day old male and female MT-KO mice had greater basal fat mass than WT mice (Fig. 1b). With SD feeding, the fat mass was greater in adult MT-KO mice than in WT mice after 42 weeks of growth (Fig. 1c, d, e). We previously reported that adipocytes derived from the dorsal-ventral subcutaneous fat pads of suckling infant MTKO mice were larger than those of WT mice [21]. These data suggested that MT negatively regulated adipose tissue formation in mice, beginning with lactation to the adult stage in both males and females under SD condition.

In the present study, in females, WT mice fed HFD showed a slightly increased body weight (Fig. 1a) compared with WT mice fed SD. Fat mass and enlargement of cell size (Fig. 2d–f) were greater in female MT-KO mice fed HFD than in WT mice fed HFD. In addition, MT-KO mice

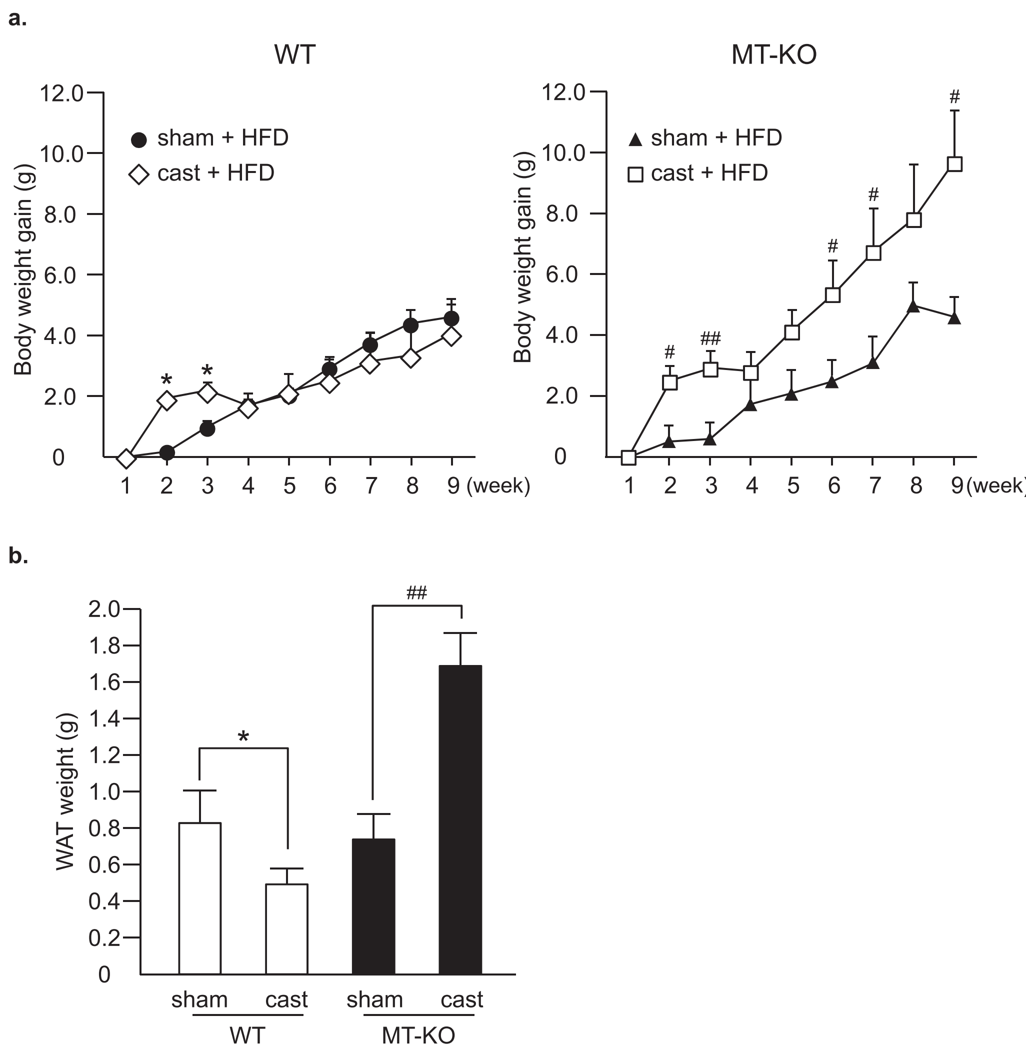

Fig. 7. Effect of surgical castration on the body weight gain and WAT weight in WT and MT-KO mice.
(a) Body weight gain; (b) WAT weight. Mice were weighed every week. Values are mean ± SEM for groups of four to six mice. Student's *t*-test was used for statistical analysis. $^{*}p < 0.05$ compared to HFD-fed sham-treated WT mice. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to HFD-fed sham-treated MT-KO mice. Both WT and MT-KO male mice were surgically castrated at six to seven weeks, then aged to 16 weeks.

fed HFD showed greater increases in body weight (Fig. 1a) and fat mass formation (Fig. 1c–e) compared with MT-KO mice fed SD in females. We calculated the number of adipocytes based on the mean adipocyte size distribution and fat pad weight. No remarkable difference in the number of adipocytes was seen between the WT and MT-KO mice. In addition, no differences in mRNA expression of regulatory factors involved in adipocyte differentiation, such as PPARγ and C/EBPs, were seen between the WT and MT-KO mice (Fig. 4). Thus, MT might not regulate the number of adipocytes and could contribute to adipocyte hypertrophy. MT may regulate adipocyte size by preventing excess lipid accumulation mediated via *Mest* and *Vldlr* (Fig. 3a–d).

Estradiol treatment of postmenopausal women for one year decreased their intra-abdominal and intrapelvic fat [22]. Ovariectomized animals have increased adiposity compared with animals with intact ovaries, but estrogen treatment in ovariectomized animals reduces fat mass and adipocyte size [23]. These data show that estrogen may have a role in preventing lipid accumulation. MT, either directly or indirectly, could promote the ability of estrogen to prevent excess lipid accumulation in obesity (Fig. 8).

Postmenopausal women with obesity who were treated with testosterone for nine months had significantly increased visceral fat compared with women receiving a placebo [24]. Further, 5α-dihydrotestosterone treatment resulted in obesity associated with reduced energy expenditure in orchidectomized mice [25]. These results suggested that androgen had an ability to increase fat mass. Androgen-associated changes in body and WAT weight are known to impact the process of obesity. Although the body weight of HFD-fed mice was comparable to that of SD-fed mice in MT-KO males, body weight (Fig. 1a) and WAT weight (Fig. 1c–e) were greatly increased by feeding HFD in male WT mice. Lindeque et al. showed that there was no statistically significant difference in body weight gain between male WT mice and MT-KO mice until only 10 weeks after HFD treatment [11]. Therefore, this observation was essentially identical to our results.

In addition, we investigated the effect of surgical castration and HFD feeding on total body weight and WAT weight. The WAT weight (Fig. 7b) in male WT castrated mice were low compared to that in male WT sham mice in the presence of androgen and MT. These results also supported that androgen had an ability to increase WAT weight in the presence of MT (Fig. 8e). To summarize, MT exaggerated the ability of androgen to increase body weight and fat mass.

In MT-KO mice fed HFD, body weight gain (Fig. 7a, right) and WAT weight (Fig. 7b) were greater in castrated mice, in the absence of both androgen and MT, than in sham mice. In addition, the increase in total body weight gain was far greater in castrated MT-KO mice fed HFD (Fig. 7a, right) than in male sham WT mice fed HFD (Fig. 7a, left). The data indicated that under the defect of MT and androgens, the

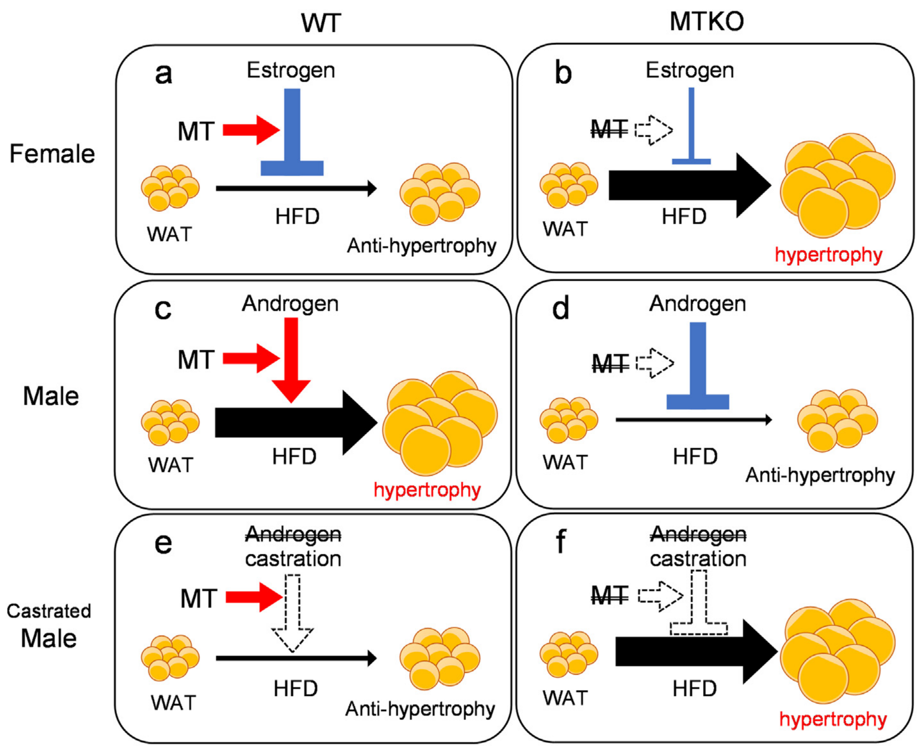

Fig. 8. Hypothetical diagram of sex inversion effects of MT expression on WAT expansion and adipocyte hypertrophy induced by HFD.
MT may have the potential to advance, directly or indirectly, two conflicting actions of sex hormones, which are the anti-hypertrophic effect of estrogen and hypertrophy-promoting effect of androgen on adipocytes in WAT of WT female and male mice, respectively (a and c). MT gene knockout may impair or reverse the sex hormone actions, resulting in female mice becoming obese and male mice avoiding obesity in HFD condition (b and d). In HFD feeding, androgen deprivation by surgical castration in males may counteract the actions of hypertrophy promotion in WT mice and anti-hypertrophy in MTKO mice (e and f). These castrated male mice show the similar phenotype as female mice corresponding to the same genotype (WT; e to a: MTKO; f to b).

cooperative regulatory role in body weight and WAT weight gain may not work. Since the castrated HFD-fed MT-KO mice exhibited the female-like phenotype in this animal study (Fig. 8f), MT may act to modify the susceptibility to obesity under sex hormones.

Although the mechanism by which testosterone regulated adipose tissue mass has not been elucidated, there were many possibilities to explain sex differences. Barbrosa-Desongles et al. showed that testosterone exerted a proliferative effect on preadipocytes that may contribute to sex differences in fat distribution [26]. ROS also accelerated adipocytic differentiation [27] and lipid accumulation [28]. Because MT had a preventive role against ROS [8], further studies on the relationship between androgen, MT and ROS in the sex difference in development of obesity are required.

The number of adipocytes increases in early life (childhood and adolescence) during WAT development, whereas it is usually constant in adults [29]. In these stages, various factors, including Cebpβ and Cebpδ, are involved in the differentiation of preadipocytes from adipocytes in the first step. The factors activate transcription of the key adipogenic regulator genes, PPARγ and C/EBPα. The abundance of mRNA for those adipocyte differentiation- and maturation-related genes was similar among all groups, regardless of diet condition, adipocyte hypertrophy, or sex (Fig. 4).

The mechanisms involved in the effects of factors, including MT, on WAT lipid accumulation are still unknown. Mest is an adipocyte enlargement factor [30], and its mRNA is expressed at the onset of the most rapid phase of fat mass expansion. VLDL-R has a primary role in the anchorage of triglyceride-rich lipoproteins, facilitating triglyceride hydrolysis by lipoprotein lipase and free fatty acid entry into tissues. In the present study, the expression of Mest and Vldlr genes in WAT is greater in female and male MT-KO mice than in WT mice (Fig. 3a and b), suggesting that MT maintains the level of expression of Mest and Vldlr in WAT. The expression of Mest and Vldlr in WAT is associated with the extent of lipid accumulation (Fig. 3c and d). These data suggest that MT has a preventive role against excess lipid accumulation in WAT via down-regulation of Mest and Vldlr. Further investigation is required to understand the regulation of their expression by MT and the relationships among these genes.

PAI-1 is the primary inhibitor of plasminogen activation in vivo, and increased concentrations of PAI-1 are observed in the plasma of obese patients [31]. Increased plasma levels of PAI-1 promote thrombosis and myocardial infarction [32]. In the present study, the expression of Serpine1 was greater in the WAT of SD-fed MT-KO mice than in SD-fed WT mice, indicating that MT could downregulate Serpine1 expression (Fig. 5), and MT-KO mice could have a greater risk of cardiovascular disease. The increased expression of Serpine1 in the WAT of male HFD-fed WT mice and female HFD-fed MT-KO mice was accompanied by increased lipid accumulation.

The present study demonstrated that MT regulated adipose tissue formation at the lactation stage and during growth in males and females. In addition, we previously reported that MT1/2 siRNA promoted lipid accumulation in 3T3-L1 adipocytes and caused proliferation of post-confluent preadipocytes [21], suggesting that MT1/2 could play a role in appropriately limiting the development of adipocytes, at least during the lactation stage and/or at the beginning of maturation. Recently, we reported that MT deficiency shortened the lifespan in male and female mice and there was sex difference in the lifespan and the aging-related physical changes of MTKO mice [15]. In addition, the insulin/insulin-like growth factor-1 signaling pathway is a well-known conserved pathway that regulates aging in several organisms. Reduction in activity of this pathway was observed to lead to extended life spans [2], and MTs regulated the adipogenic differentiation of preadipocytes via the insulin signaling pathway [21]. Moreover, MT expression was increased in long-lived mutants [33,34]. MT deficiency in mice could accelerate aging though activation of the above pathway. Thus, MT could, at the minimum, influence life span via its action at two stages of adipose tissue development and in insulin signaling.

In conclusion, MT appears to have three effects on fat mass development of WAT. First, MT has a common role in both male and female mice in maintaining adipocyte lipids during lactation and growth. Second, MT prevents excess lipid accumulation and adipocyte hypertrophy in cooperation with estrogen in HFD-fed female mice (Fig. 8a and b). Finally, MT enhances the action of androgen to increase WAT weight and adipocyte size in HFD-fed male mice (Fig. 8c). In addition, androgen without the cooperation of MT does not only weaken, but reversely suppresses the fat accumulation potential of WAT in male mice (Fig. 8d). Indeed, defects of both androgen and MT lead to higher body weight gain and WAT weight of castrated male MT-KO mice compared to the sham MT-KO group. Taken together, MT may modify the action of sex hormones concerning fat accumulation and body weight gain. Further detailed studies on the relationship between MT and sex hormones would be beneficial.

Acknowledgments

The authors thank M. Inoue (Tokushima Bunri University, Tokushima, Japan) for assistance in histology and M. Mita (Kitasato University, Tokyo, Japan) for mouse husbandry. This work was supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (1430767; to M. S., and 21590144 to T. K), by the Science Research Promotion Fund for Private University (T. K.), and by the Open Research Center Fund for Promotion.

Author contributions

All authors contributed equally to this work. M. S., T. K and S. S. designed the study; S. T, D. F., and T. K. performed the experiments; M. S., T. K. and S. T. collected and analyzed the data; T. K. and S. T. provided mice and reagent; and M. S., T. K., Y. K., and S. S. wrote the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

[1] S.K. Garg, H. Maurer, K. Reed, R. Selagamsetty, Diabetes and cancer: two diseases with obesity as a common risk factor, Diabetes Obes. Metab. 16 (2) (2014) 97–110.
[2] C. Kenyon, The plasticity of aging: insights from long-lived mutants, Cell 120 (4) (2005) 449–460.
[3] M. Bluher, B.B. Kahn, C.R. Kahn, Extended longevity in mice lacking the insulin receptor in adipose tissue, Science 299 (5606) (2003) 572–574.
[4] A.K. Palmer, J.L. Kirkland, Aging and adipose tissue: potential interventions for diabetes and regenerative medicine, Exp. Gerontol. 86 (2016) 97–105.
[5] S.R. Davis, R.J. Cousins, Metallothionein expression in animals: a physiological perspective on function, J. Nutr. 130 (5) (2000) 1085–1088.
[6] M. Sato, I. Bremner, Oxygen free radicals and metallothionein, Free Radic. Biol. Med. 14 (3) (1993) 325–337.
[7] M. Sato, M. Kondoh, Recent studies on metallothionein: protection against toxicity of heavy metals and oxygen free radicals, Tohoku J. Exp. Med. 196 (1) (2002) 9–22.
[8] F. Haq, M. Mahoney, J. Koropatnick, Signaling events for metallothionein induction, Mutat. Res. 533 (1–2) (2003) 211–226.
[9] M. Kondoh, M. Tsukada, M. Kuronaga, M. Higashimoto, M. Takiguchi, S. Himeno, Y. Watanabe, M. Sato, Induction of hepatic metallothionein synthesis by endoplasmic reticulum stress in mice, Toxicol. Lett. 148 (1–2) (2004) 133–139.
[10] J.H. Beattie, A.M. Wood, A.M. Newman, I. Bremner, K.H. Choo, A.E. Michalska, J.S. Duncan, P. Trayhurn, Obesity and hyperleptinemia in metallothionein (-I and -II) null mice, Proc. Natl. Acad. Sci. U. S. A. 95 (1) (1998) 358–363.
[11] J.Z. Lindeque, P.J. Jansen van Rensburg, R. Louw, F.H. van der Westhuizen, S. Florit, L. Ramirez, M. Giralt, J. Hidalgo, Obesity and metabolomics: metallothioneins protect against high-fat diet-induced consequences in metallothionein knockout mice, OMICS 19 (2) (2015) 92–103.
[12] M. Sato, T. Kawakami, M. Kondoh, M. Takiguchi, Y. Kadota, S. Himeno, S. Suzuki, Development of high-fat-diet-induced obesity in female metallothionein-null mice, FASEB J. 24 (7) (2010) 2375–2384.
[13] K. Karastergiou, S.R. Smith, A.S. Greenberg, S.K. Fried, Sex differences in human adipose tissues - the biology of pear shape, Biol. Sex Differ. 3 (1) (2012) 13.
[14] A.E. Newell-Fugate, The role of sex steroids in white adipose tissue adipocyte function, Reproduction 153 (4) (2017) R133–R149.
[15] Y. Kadota, Y. Aki, Y. Toriuchi, Y. Mizuno, T. Kawakami, M. Sato, S. Suzuki, Deficiency of metallothionein-1 and -2 genes shortens the lifespan of the 129/Sv mouse strain, Exp. Gerontol. 66 (2015) 21–24.
[16] B.A. Masters, E.J. Kelly, C.J. Quaife, R.L. Brinster, R.D. Palmiter, Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium, Proc. Natl. Acad. Sci. U. S. A. 91 (2) (1994) 584–588.
[17] J.P. Chua, S.L. Reddy, Z. Yu, E. Giorgetti, H.L. Montie, S. Mukherjee, J. Higgins, R.C. McEachin, D.M. Robins, D.E. Merry, J.A. Iniguez-Lluhi, A.P. Lieberman, Disrupting SUMOylation enhances transcriptional function and ameliorates polyglutamine androgen receptor-mediated disease, J. Clin. Invest. 125 (2) (2015) 831–845.
[18] T. Kawakami, N. Hanao, K. Nishiyama, Y. Kadota, M. Inoue, M. Sato, S. Suzuki, Differential effects of cobalt and mercury on lipid metabolism in the white adipose tissue of high-fat diet-induced obesity mice, Toxicol. Appl. Pharmacol. 258 (1) (2011) 32–42.
[19] M.S. Do, S.Y. Nam, S.E. Hong, K.W. Kim, J.S. Duncan, J.H. Beattie, P. Trayhurn, Metallothionein gene expression in human adipose tissue from lean and obese subjects, Horm. Metab. Res. 34 (6) (2002) 348–351.
[20] P. Trayhurn, J.S. Duncan, A.M. Wood, J.H. Beattie, Metallothionein gene expression and secretion in white adipose tissue, Am. J. Physiol. Regul. Integr. Comp. Physiol. 279 (6) (2000) R2329–R2335.
[21] Y. Kadota, Y. Toriuchi, Y. Aki, Y. Mizuno, T. Kawakami, T. Nakaya, M. Sato, S. Suzuki, Metallothioneins regulate the adipogenic differentiation of 3T3-L1 cells via the insulin signaling pathway, PLoS One 12 (4) (2017) e0176070.
[22] I. Mattiasson, M. Rendell, C. Tornquist, S. Jeppsson, U.L. Hulthen, Effects of estrogen replacement therapy on abdominal fat compartments as related to glucose and lipid metabolism in early postmenopausal women, Horm. Metab. Res. 34 (10) (2002) 583–588.
[23] T.M. D'Eon, S.C. Souza, M. Aronovitz, M.S. Obin, S.K. Fried, A.S. Greenberg, Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways, J. Biol. Chem. 280 (43) (2005) 35983–35991.
[24] J.C. Lovejoy, G.A. Bray, M.O. Bourgeois, R. Macchiavelli, J.C. Rood, C. Greeson, C. Partington, Exogenous androgens influence body composition and regional body fat distribution in obese postmenopausal women—a clinical research center study, J. Clin. Endocrinol. Metab. 81 (6) (1996) 2198–2203.
[25] S. Moverare-Skrtic, K. Venken, N. Andersson, M.K. Lindberg, J. Svensson, C. Swanson, D. Vanderschueren, J. Oscarsson, J.A. Gustafsson, C. Ohlsson, Dihydrotestosterone treatment results in obesity and altered lipid metabolism in orchidectomized mice, Obesity (Silver Spring) 14 (4) (2006) 662–672.
[26] A. Barbosa-Desongles, C. Hernandez, R. Simo, D.M. Selva, Testosterone induces cell proliferation and cell cycle gene overexpression in human visceral preadipocytes, Am. J. Physiol. Cell Physiol. 305 (3) (2013) C355–C359.
[27] H. Lee, Y.J. Lee, H. Choi, E.H. Ko, J.W. Kim, Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion, J. Biol. Chem. 284 (16) (2009) 10601–10609.
[28] M. Sekiya, A. Hiraishi, M. Touyama, K. Sakamoto, Oxidative stress induced lipid accumulation via SREBP1c activation in HepG2 cells, Biochem. Biophys. Res. Commun. 375 (4) (2008) 602–607.
[29] K.L. Spalding, E. Arner, P.O. Westermark, S. Bernard, B.A. Buchholz, O. Bergmann, L. Blomqvist, J. Hoffstedt, E. Naslund, T. Britton, H. Concha, M. Hassan, M. Ryden, J. Frisen, P. Arner, Dynamics of fat cell turnover in humans, Nature 453 (7196) (2008) 783–787.
[30] L. Nikonova, R.A. Koza, T. Mendoza, P.M. Chao, J.P. Curley, L.P. Kozak, Mesoderm-specific transcript is associated with fat mass expansion in response to a positive energy balance, FASEB J. 22 (11) (2008) 3925–3937.
[31] V. Salomaa, V. Stinson, J.D. Kark, A.R. Folsom, C.E. Davis, K.K. Wu, Association of fibrinolytic parameters with early atherosclerosis. The ARIC Study. Atherosclerosis Risk in Communities Study, Circulation 91 (2) (1995) 284–290.
[32] I. Shimomura, T. Funahashi, M. Takahashi, K. Maeda, K. Kotani, T. Nakamura, S. Yamashita, M. Miura, Y. Fukuda, K. Takemura, K. Tokunaga, Y. Matsuzawa, Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity, Nat. Med. 2 (7) (1996) 800–803.
[33] W.R. Swindell, Metallothionein and the biology of aging, Ageing Res. Rev. 10 (1) (2011) 132–145.
[34] E. Mocchegiani, L. Costarelli, A. Basso, R. Giacconi, F. Piacenza, M. Malavolta, Metallothioneins, ageing and cellular senescence: a future therapeutic target, Curr. Pharm. Des. 19 (9) (2013) 1753–1764.