

# Tissue inhibitor of matrix metalloproteinase 1 (TIMP1) controls adipogenesis in obesity in mice and in humans

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## Abstract

**Aims/hypothesis** Extracellular matrix reorganisation is a crucial step of adipocyte differentiation and is controlled by the matrix metalloproteinase–tissue inhibitor of matrix metalloproteinase (TIMP) enzyme system. We therefore sought to define the role of TIMP1 in adipogenesis and to elucidate whether upregulation of TIMP1 in obesity has direct effects on adipocyte formation.

**Methods** TIMP1 protein levels and mRNA were measured in lean and obese mice with a focus on levels in adipose tissue. We also analysed the effect of recombinant murine TIMP1 on adipogenesis, adipocyte size and metabolic control in vitro and in vivo.

**Results** TIMP1 levels were increased in the serum and adipose tissue of obese mouse models. Recombinant murine TIMP1 inhibited adipocyte differentiation in 3T3-L1 as well as in subcutaneous primary pre-adipocytes. Conversely, neutralising TIMP1 with a specific antibody enhanced adipocyte differentiation. In vivo, injection of recombinant TIMP1 in mice challenged with a high-fat diet led to enlarged adipocytes. TIMP1-treated mice developed an impaired metabolic profile with increased circulating NEFA levels, hepatic triacylglycerol accumulation and accelerated insulin resistance. Altered glucose clearance in TIMP1-injected mice was due to changes in adipose tissue

glucose uptake, whereas muscle glucose clearance remained unaffected.

**Conclusions/interpretation** TIMP1 is a negative regulator of adipogenesis. In vivo, TIMP1 leads to enlarged adipocytes in the state of overnutrition. This might contribute to the detrimental metabolic consequences seen in TIMP1-injected mice, such as systemic fatty acid overload, hepatic lipid accumulation and insulin resistance.

**Keywords** Adipocyte hypertrophy · Adipogenesis · Diet-induced obesity · Insulin resistance · TIMP1 · Tissue inhibitor of metalloproteinase 1

## Abbreviations

MMP Matrix metalloproteinase  
SVF Stromal–vascular fraction of adipose tissue  
TIMP Tissue inhibitor of matrix metalloproteinase

## Introduction

Obesity is the main risk factor for type 2 diabetes and has become a growing health problem in the last decades [1–3]. Expansion of adipose tissue results from an imbalance between energy intake and energy expenditure. Secondary complications caused by obesity partly depend on how well adipose tissue can adapt to an extended nutrient supply [4, 5]. One way of buffering excess nutrients in adipose tissue is the generation of fat cells through de novo differentiation. This hyperplastic response is preferable to a hypertrophic reaction, where nutrients are stored in pre-existing adipocytes. Adipocyte hypertrophy commonly leads to enlarged, insulin-resistant fat cells with a high lipolytic rate, thus promoting ectopic

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lipid accumulation and systemic insulin resistance [6–9]. New adipocytes can differentiate from precursor cells, which reside in the stromal–vascular fraction of adipose tissue (SVF) [10, 11]. For a fibroblast-like pre-adipocyte to become a lipid-laden adipocyte, restructuring of the extracellular matrix is indispensable [12–15]. Therefore, extracellular matrix remodelling has an important impact on adipocyte differentiation [16, 17]. The concomitant extracellular protein degradation is mediated by matrix metalloproteinases (MMPs) and their regulatory counterparts, the tissue inhibitors of matrix metalloproteinases (TIMPs) [17].

Altered levels of MMPs have been observed in adipose tissue of obese participants and the MMP system is tightly regulated during adipogenesis [18]. MMPs influence the process of adipogenesis in a repressive or activating way depending on substrate specificity [17]. Mice deficient in MMP3, MMP11 or MMP19 exhibit increased adiposity during diet-induced obesity [19–21]. In female mice after lactation MMPs also influence adipogenesis in the mammary gland. Loss of MMP3, for example, leads to increased fat accumulation [22]. Moreover, lack of plasminogen, a precursor of plasmin, a key enzyme activating MMPs, blocks adipocyte differentiation [23]. Finally, mice treated with a global MMP inhibitor display reduced adipose tissue weight concomitant with a lower total number of adipocytes when exposed to a high-fat diet [24]. This is consistent with the fact that the global MMP inhibitor BB-94 inhibits 3T3-L1 adipocyte differentiation [18].

MMPs are inhibited in 1:1 stoichiometry by the family of TIMP proteins. TIMP1 inhibits most MMPs except MMP14, MMP16 and MMP19 [25]. Analysis in adipose tissue of lean and obese mice, and humans revealed significant upregulation of TIMP1 in obesity [18, 26]. Conflicting data have been reported concerning the functional role of TIMP1 in adipose tissue. Whereas male TIMP1-deficient mice kept on a high-fat diet develop less adiposity, female TIMP1-deficient mice accumulate more adipose tissue due to hyperphagia [27, 28]. On the other hand, systemic adenoviral overproduction of the human TIMP1 homologue in mice had no effect on adipose tissue mass [29]. In vitro, human TIMP1 did not influence adipogenesis of murine 3T3-F442A cells, whereas in another study, human TIMP1 accelerated 3T3-L1 differentiation [22, 29]. A certain drawback here is the use of human TIMP1 in mouse model systems, which might disturb the interaction between MMPs and their inhibitors due to cross species differences.

As TIMP1 levels are upregulated in obesity, the aim of this study was to define the role of TIMP1 in adipocyte formation and its potential influence on metabolism in the state of obesity.

## Methods

**Real-time PCR** cDNA was produced using a cDNA kit (Multi-MACS; Miltenyi, Bergisch Gladbach, Germany). mRNA expression was assessed using SybrGreen (Invitrogen, Basel, Switzerland) and normalised to *Rplp0*.

Primers (annealing temperature 60°C): *Rplp0* forward GCCGTGATGCCAGGGAAGACA, *Rplp0* reverse CATCTGCTT GGAGCCCACGTTG; *Timp1* forward GATATGCCACAAGTCCCAGAACC, *Timp1* reverse GCACACCCACAGCCAGCACTAT; *Pparg* (also known as *Pparg*) forward AGGCGAGGGCGATCTTGACAG, *Pparg* reverse AATTCGGATGGCCACCTCTTTG; *A-Fabp* (also known as *Fabp4*) forward ATGAAATCACCGCA GACGACAG, *A-Fabp* reverse GCCTTTCATAAC ACATTCCACCAC.

**TIMP1 secretion from SVF** SVF and adipocytes were essentially isolated as described previously [30]. Adipose tissue was digested for 1 h with 2 mg/ml collagenase type II. Adipocytes were cultured in 24-well 0.4 µm filter inserts (Becton Dickinson, Franklin Lakes, NJ, USA). SVF was filtered through a 40 µm net and erythrocytes were lysed. Cells were seeded on collagen-coated plates at 80% density in high-glucose DMEM with 10% (vol./vol.) fetal bovine serum and penicillin/streptomycin. The next day, the medium was changed to Krebs–Ringer buffer and collected after 24 h. TIMP1 in the supernatant fraction was measured using an ELISA kit (Quantikine Mouse TIMP1; R&D Systems, Minneapolis, MN, USA).

**MMP activity assays** Inhibition of MMP9 (Anaspec, Fremont, CA, USA) by TIMP1 was tested using a kit (SensoLyte 570 Generic MMP Assay Fluorimetric; Anaspec, Fremont, CA, USA). Human or murine MMP9 was used at 30 ng/ml corresponding to enzyme:substrate ratios of 1:43 to 1:350. Human TIMP1 was purchased from R&D Systems (R&D Systems).

For global MMP activity from adipose tissue extracts, frozen adipose tissue was dounced in 300 µl hypotonic buffer (10 mmol/l KCl, 1.5 mmol/l MgCl<sub>2</sub>, 10 mmol/l HEPES pH 7.9), and 375 µl hypertonic buffer (20 mmol/l HEPES/KOH pH 7.9, 25% (vol./vol.) glycerol, 0.2 mmol/l EDTA, 1.5 mmol/l MgCl<sub>2</sub>, 1.2 mol/l NaCl) was added, followed by incubation at 4°C for 30 min. Cleared lysates (50 µl) were tested for MMP activity using the SensoLyte kit (Anaspec, Fremont, CA, USA).

**Recombinant murine *Timp1*** Murine *Timp1* was cloned from adipose tissue cDNA and ligated into the pSecTagA vector (Invitrogen, Basel, Switzerland) using the following primers: murine *Timp1* forward AAGCTTTTGTAG

CTGTGCCCCACCCCA, reverse CTCGAGTCGGGC CCCAAGGGATCTCC. For protein production HEK293T cells were transfected with lipofectamine (Invitrogen, Basel, Switzerland). Opti-MEM supernatant fraction was collected every 2 days. Murine TIMP1 was purified twice via the his-tag using a Ni-NTA column (GE Healthcare, Glattbrugg, Switzerland) connected to an automated fast protein liquid chromatography system. Murine TIMP1 was dialysed against PBS and analysed by western blot using anti-his tag antibody (Cell Signaling, Danvers, MA, USA) or Coomassie-stained 12% (wt/vol.) SDS-polyacrylamide gel.

**Adipocyte differentiation** 3T3-L1 pre-adipocytes and primary cells from SVF were cultured on collagen-coated black 96-well plates in DMEM supplemented with 10% (vol./vol.) fetal bovine serum and penicillin/streptomycin. Adipogenesis was induced by treating 48 h post-confluent 3T3-L1 pre-adipocytes or 80% confluent stromal-vascular cells with induction medium (1 µg/ml insulin, 1 µmol/l dexamethasone, 115 µg/ml isobutylmethylxanthine) for 2 days, followed by 2 days of insulin medium (1 µg/ml) and 2 days of normal medium. Murine TIMP1 at indicated concentrations, monoclonal rat anti-mouse TIMP1 antibody (R&D Systems) or rat IgG1 isotype control antibody (MBL, Woburn, MA, USA) at 0.5 µg/ml were added to the medium 24 h after cell seeding until the third day of differentiation. Differentiated cells were fixed with 5% (wt/vol.) formaldehyde and stained with 4 µmol/l Hoechst (nuclei), 5 µmol/l Syto60 (cytosol) and 2 µmol/l Bodipy (lipid droplets) or Oil Red O. We took nine pictures per well with an automated microscope (CellWorx, Thermo Fisher Scientific, Pittsburgh, PA, USA). Pictures were analysed using Cell Profiler Software [31].

**Adipose tissue western blots** Adipose tissue extracts were prepared as described under MMP activity assays. Protein concentration was determined using the DC protein assay (BioRad, Reinach, Switzerland). Tissue lysates were subjected to 12% (wt/vol.) SDS-polyacrylamide gel electrophoresis and western blotting using the following antibodies: goat anti-mouse/rat MMP2 (1:1,000), goat anti-mouse MMP9 (1:1,000; R&D Systems), mouse anti-mouse  $\gamma$ -tubulin (1:5,000; Sigma-Aldrich, Buchs, Switzerland).

**TIMP1 levels in human obese participants** TIMP1 serum levels were measured in 20 obese men (BMI 32.9–54.9 kg/m<sup>2</sup>) using a kit (human TIMP1 Quantikine ELISA; R&D Systems). All patients enrolled in the present study gave informed consent to participate. The study was approved by the ethical board of the University Hospital of Heidelberg, Germany.

**Animal studies** All animal studies were approved by the veterinary office of Zürich. Male C57BL/6J mice were kept on a 12/12 h light/dark cycle in a pathogen-free animal facility. C57BL/6J-Lep<sup>ob</sup> mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). For diet-induced obesity, mice were fed a high-fat diet (Provimi Kliba, Kaiseraugst, Switzerland) containing 60% energy from fat. High-fat diet was given for 14 weeks (tissue expression and secretion studies) or 8 weeks (TIMP1 serum concentrations). Control animals on a regular chow diet were killed at the corresponding age. For murine TIMP1 injection studies, subcutaneous murine TIMP1 (200 µg/kg) or PBS injections were administered daily into the scapular region of male C57BL/6J mice (age 6–8 weeks) for 3 weeks, during which mice were fed a high-fat diet. For analysis of short-term effects, mice were injected with murine TIMP1 and fed a high-fat diet for three consecutive days, followed by metabolism cage analysis of oxygen consumption ( $\dot{V}O_2$ ), carbon dioxide production ( $\dot{V}CO_2$ ), food intake and physical activity using a metabolism cage system (Oxymax; Columbus Instruments, Columbus, OH, USA).

**Serum measurements** Blood glucose was measured with a glycometer (Contour, Bayer HealthCare, Zürich, Switzerland). For the insulin tolerance test, insulin (0.75 U/kg of body weight) was injected after 3 weeks of high-fat diet and murine TIMP1 injections. Insulin was determined with a kit (Rat/Mouse Insulin ELISA; Crystal Chem, Downers Grove, IL, USA). Triacylglycerol and NEFA were measured with the following kits: Trig/GB (Roche/Hitachi, Basel, Switzerland); and NEFA-HR kit (Wako, Neuss, Germany). TIMP1 serum levels were assessed with a kit (Quantikine Mouse TIMP1 ELISA; R&D Systems).

**Liver triacylglycerol** Lipids from 150 mg liver were extracted with 1 ml hexane:isopropanol (3:2) by homogenising the tissue in a TissueLyser. Supernatant fraction was taken off and the extraction repeated with 0.5 ml hexane/isopropanol. We then added 0.5 ml 15% (wt/vol.) Na<sub>2</sub>SO<sub>4</sub> and evaporated the upper organic phase overnight. Lipids were dissolved in 2 ml Triton X-100/methanol/butanol (1:1:3) and measured with the Trig/GB Kit (Roche/Hitachi, Basel, Switzerland).

**Adipocyte and fat pad size** Adipose tissue was fixed overnight in 5% (wt/vol.) paraformaldehyde before paraffin embedding and sectioning to 10 µm slices. Haematoxylin and eosin staining of sections was performed according to standard procedures [32]. Microscopic pictures were taken and cell size analysed using Cell Profiler Software [31]. Fat pads were measured with an animal computed tomography scanner (LaTheta; Echo Medical Systems, Houston, TX, USA).

**Ex vivo glucose uptake** Ex vivo glucose uptake was performed essentially as described previously [33]. After a 6 h fast during the dark period, extensor digitorum longus muscles and adipose tissue were dissected and incubated for 20 min at 37°C in KRB buffer containing 0.1% (wt/vol.) BSA, 5 mmol/l HEPES and 10 mmol/l glucose with or without 10 nmol/l insulin. Tissues were rinsed and glucose transport assessed during 75 min at 37°C using 2-deoxy[<sup>14</sup>C]glucose (0.07 MBq; Perkin Elmer, Schwerzenbach, Switzerland) in KRB with or without insulin. Tissues were incubated for another 10 min at 37°C in KRB to remove extracellular radiolabelled glucose. Tissues were incubated overnight at –20°C in 1 mol/l NaOH. Samples were dounced and centrifuged at 13,000 g for 10 min. Radioactivity was determined by liquid scintillation counting (Beckman Coulter, Brea, CA, USA) using a liquid scintillation cocktail (Ultima Gold; Perkin Elmer).

**Statistical analysis** Results are given as mean ± standard deviation. Statistical analyses were performed using a two-tailed Student's *t* test.

## Results

It is known that obesity leads to a decreased capacity of compensatory de novo adipogenesis [34, 35], which is influenced by paracrine or endocrine factors. We therefore analysed whether *Timp1* mRNA expression was deregulated in mouse models of obesity. In mice with diet-induced obesity mRNA expression of *Timp1* was upregulated in several tissues, including liver, kidney, heart, colon, thymus and brain. The most prominent increase in *Timp1* mRNA expression was seen in adipose tissue (Fig. 1a). These data were corroborated against TIMP1 protein levels in adipose tissue and heart muscle. TIMP1 protein levels were increased two- to fourfold in subcutaneous and visceral adipose tissue of obese compared with lean mice, whereas TIMP1 protein in heart muscle was only mildly affected. TIMP1 protein was generally more abundant in visceral than in subcutaneous adipose tissue compartments (Fig. 1b). As TIMP1 is a circulating factor, we also measured TIMP1 in the serum of lean and obese mice. In *ob/ob* mice and mice with diet-induced obesity, TIMP1 serum concentrations were upregulated three- to fourfold compared with lean mice (Fig. 1c).

To get further insight into TIMP1 secretion from adipose tissue, protein secretion was measured in different fat depots and cell fractions. TIMP1 secretion was higher in visceral than in subcutaneous adipose tissue (Fig. 1d). TIMP1 secretion was also higher from SVF than from adipocytes, though direct comparisons between adherent

SVF and adipocytes based on cellular protein content are difficult. TIMP1 secretion of SVF from mice fed a high-fat diet for 14 weeks did not differ compared with SVF from lean mice. However, adipocytes from animals on high-fat diet showed higher levels of TIMP1 secretion than those from mice fed a standard chow diet (Fig. 1d).

As it has been reported that TIMP1 serum levels are higher in obese than lean patients [26], we analysed whether TIMP1 serum levels in participants with severe obesity also correlated with type 2 diabetes. In an obese cohort of 20 men (BMI 32.9–54.9 kg/m<sup>2</sup>), there was a significant positive correlation between TIMP1 serum levels and HOMA index, whereas BMI did not correlate with circulating TIMP1 levels in these obese patients (Fig. 1e, f). In female patients there was no significant correlation (data not shown).

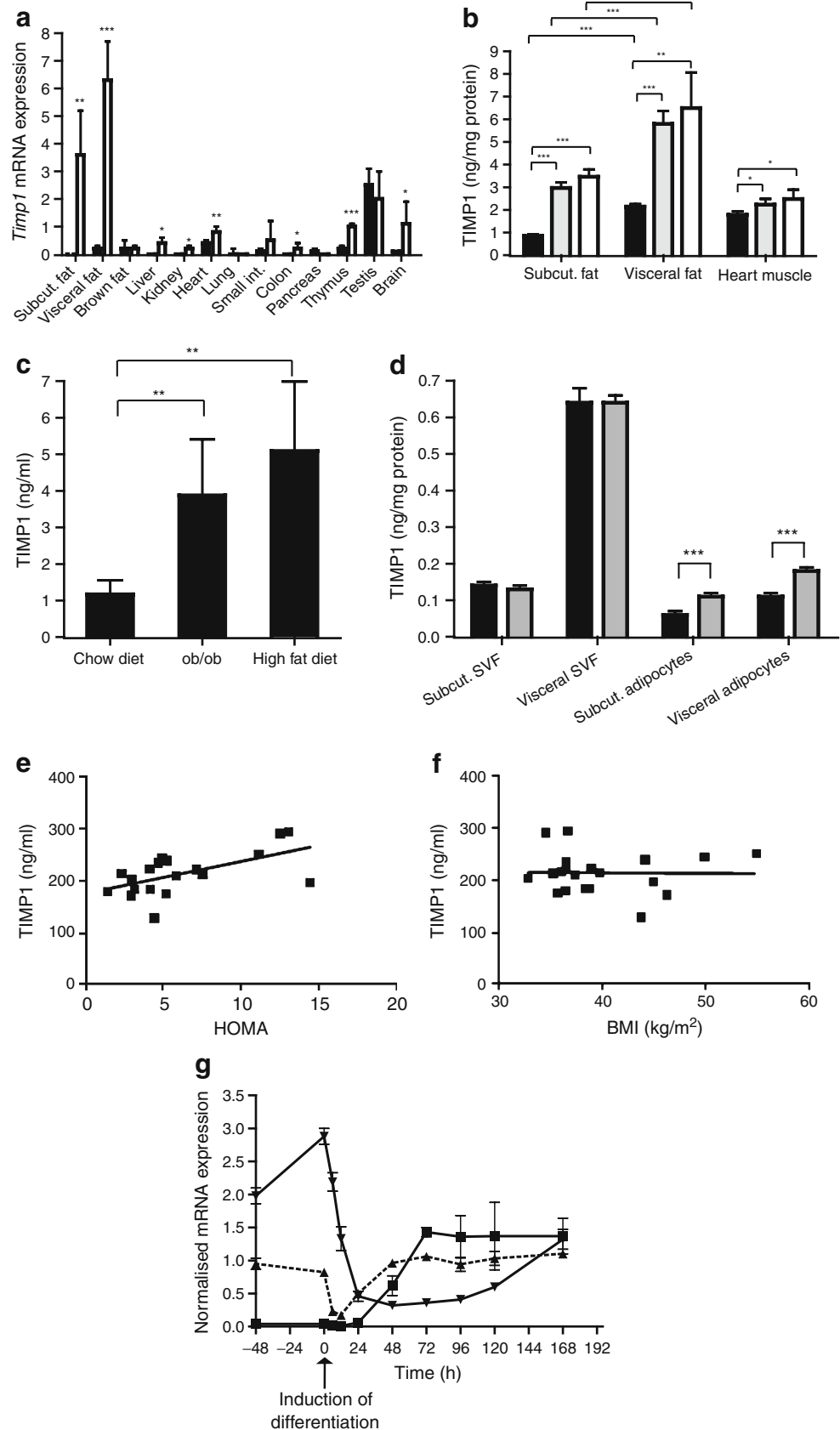
Whereas TIMP1 serum levels also increased in mice in the context of obesity (Fig. 1c), TIMP1 serum levels in a mouse model of type 1 diabetes (streptozotocin-induced) did not differ compared with control mice. In summary, these results indicate that the increase in TIMP1 serum levels is predominantly associated with obesity.

To shed light on a possible role of TIMP1 in adipogenesis, we analysed *Timp1* mRNA expression in the course of 3T3-L1 adipocyte differentiation. Expression of *Timp1* mRNA dropped at the time of induction and was very low in post-confluent differentiating 3T3-L1 pre-adipocytes (Fig. 1g).

To test whether murine TIMP1 also plays a functional role in adipogenesis, we purified recombinant mouse TIMP1 (murine TIMP1) from the cell culture supernatant fraction (Fig. 2a). As seen in Fig. 2b, c, increasing amounts of murine TIMP1 added to the medium in the range of endogenously secreted TIMP1 from 3T3-L1 pre-adipocytes (1.27±0.14 µg/ml) led to a progressive decrease in 3T3-L1 adipocyte formation. We next tested whether this inhibitory effect could also be seen in primary cells from murine adipose SVF. Whereas addition of murine TIMP1 did not change the differentiation capacity of visceral SVF, it did lower the degree of differentiation in subcutaneous SVF (Fig. 3a–c). The opposite effect was achieved using a neutralising antibody against TIMP1, which increased adipocyte differentiation in subcutaneous SVF only (Fig. 3d,e).

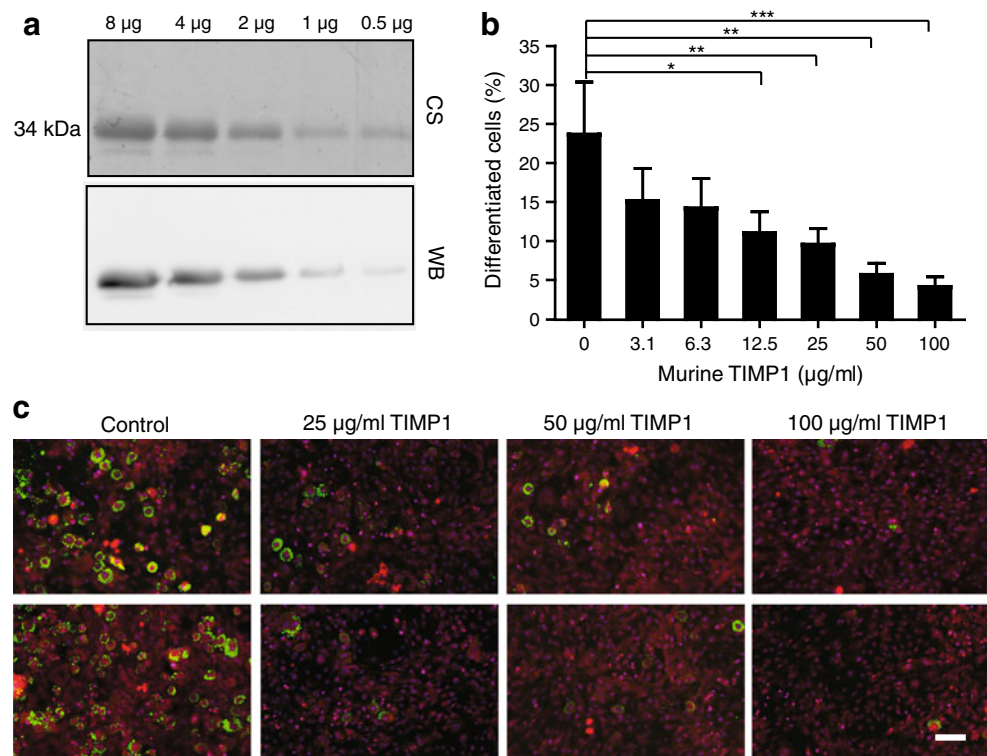
Previous reports using the human analogue of TIMP1 (human TIMP1) in cell culture models of adipogenesis did not observe any influence on adipocyte differentiation. As human and mouse TIMP1 only share 72% homology, we hypothesised that the human and murine isoforms of TIMP1 inhibit MMPs to differing degrees. We therefore compared the ability of human TIMP1 to inhibit murine MMP9 or human MMP9 in an MMP activity assay. As shown in Fig. 3f, human TIMP1 inhibited proteolytic

**Fig. 1** TIMP1 changes in obesity. **a** Tissue mRNA expression of *Timp1* in male C57Bl/6J mice on chow diet (black bars) or after 14 weeks of high-fat diet (white bars). Expression was normalised to *Rplp0*;  $n=5$ . **b** TIMP1 protein levels in tissue extracts of subcutaneous fat, visceral fat and heart muscle from male C57Bl/6J mice on chow diet (black bars) or high-fat diet (grey bars) or crossed into the *ob/ob* background (white bars). TIMP1 protein concentration was measured by ELISA and normalised to total protein concentration;  $n=4$ . **c** TIMP1 protein concentrations in serum of lean and obese mice measured by ELISA;  $n=5$ . **d** TIMP1 protein secretion from murine SVF or adipocytes of C57Bl/6J mice on chow diet (black bars) or high-fat diet (grey bars). Supernatant fractions were collected after 24 h and TIMP1 concentrations measured by ELISA;  $n=5$ . **e** Human TIMP1 serum concentrations in obese men plotted against HOMA index or **(f)** BMI;  $n=20$ . **e**  $R^2=0.34$ ,  $p=0.009$ ; **(f)**  $R^2=0.00$ ,  $p=0.96$ . **g** mRNA expression of *Timp1* (inverted triangles), *Ppar $\gamma$*  (triangles) and *A-Fabp* (squares) normalised to *Rplp0* in the course of 3T3-L1 differentiation;  $n=5$ . Values represent mean  $\pm$  SD; \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$



**Fig. 2** TIMP1 inhibits 3T3-L1 adipocyte differentiation.

**a** Coomassie staining (CS) and western blot (WB) of purified recombinant murine TIMP1. Murine TIMP1 was detected using an anti his-tag antibody. **b** 3T3-L1 pre-adipocytes were differentiated into adipocytes in the presence of different amounts of murine TIMP1 as labelled. Lipid droplet formation was assessed by fluorescent staining with automated image acquisition and analysis;  $n=4$ . Values represent mean  $\pm$  SD; \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ . **c** Representative images of differentiated 3T3-L1 adipocytes, with treatments as indicated. Green, lipid droplets; blue, nuclei; red, cytoplasm. Scale bar 100  $\mu\text{m}$



activity of human MMP9 more efficiently than that of murine MMP9. In addition, we analysed the efficacy of human and murine TIMP1 to inhibit murine MMP9 activity. As seen in Fig. 3g, murine TIMP1 showed a higher inhibition of murine MMP9 than of human TIMP1.

To analyse whether murine TIMP1 also affected adipocyte plasticity in vivo we treated male C57BL/6J mice fed a high-fat diet with murine TIMP1. Treatment involved daily injection of 200  $\mu\text{g/kg}$  body weight of murine TIMP1 or PBS into subcutaneous, scalpal adipose tissue for a time period of 3 weeks. Apart from local accumulation in the subcutaneous fat, this injection regime also led to increased circulating blood levels of TIMP1 approximately threefold over baseline levels (Fig. 4a). To assess the effect of murine TIMP1 injection on adipose tissue development, we measured whole body weight as well as fat pad weight by computed tomography scanning (Fig. 4b, c). Body and fat pad weight gain did not differ between PBS-injected and murine TIMP1-injected mice. However, we did observe an increase in mean adipocyte size and a shift in the size distribution towards larger adipocytes in mice injected with murine TIMP1 (Fig. 4d–f). This is in line with the observation that murine TIMP1 inhibits adipocyte formation, thus causing adipocyte hypertrophy.

Moreover, mice injected with murine TIMP1 showed higher blood glucose and insulin levels, as well as decreased insulin sensitivity in an insulin tolerance test compared with control animals, indicating increased insulin resistance (Fig. 5a–c). In line with this, NEFA serum

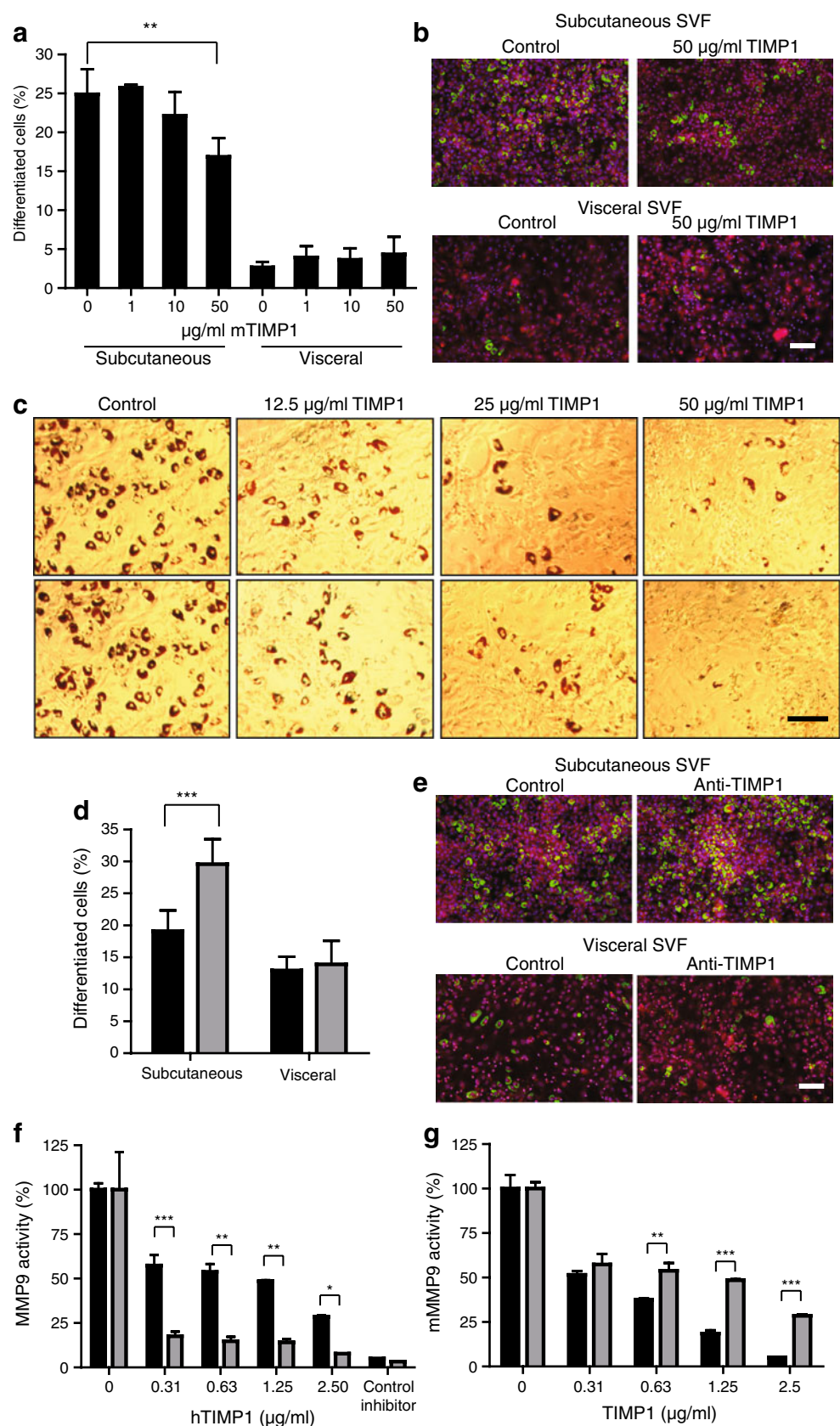
concentrations and liver triacylglycerol content were increased in mice treated with murine TIMP1 (Fig. 5d,e), whereas serum triacylglycerol levels were unchanged (Fig. 5f). To analyse whether murine TIMP1 injections altered glucose uptake specifically in adipose tissue, we performed ex vivo glucose uptake studies in fat and muscle from mice that had been injected with murine TIMP1 or PBS for 3 weeks in combination with a high-fat diet. As seen in Fig. 5g, murine TIMP1 injections led to decreased insulin-stimulated glucose uptake predominantly into subcutaneous and, to a minor extent, into visceral adipose tissue, whereas muscle glucose uptake was unaffected.

To elucidate whether murine TIMP1 injections influenced MMP levels in adipose tissue, we analysed production of MMP2 and MMP9, which are known to inhibit adipogenesis [36]. Although MMP levels varied between mice, we saw a general trend towards upregulation of MMP, with higher production of the inactive pro-form of MMP9 and MMP2 in mice injected with murine TIMP1 (Fig. 6a–f). These data were corroborated against MMP activity in adipose tissue extracts. As seen in Fig. 6g, murine TIMP1 injections led to a significant decrease in global MMP activity in subcutaneous as well as visceral adipose tissue. Interestingly, global MMP activity as such was considerably lower in visceral than in subcutaneous adipose tissue.

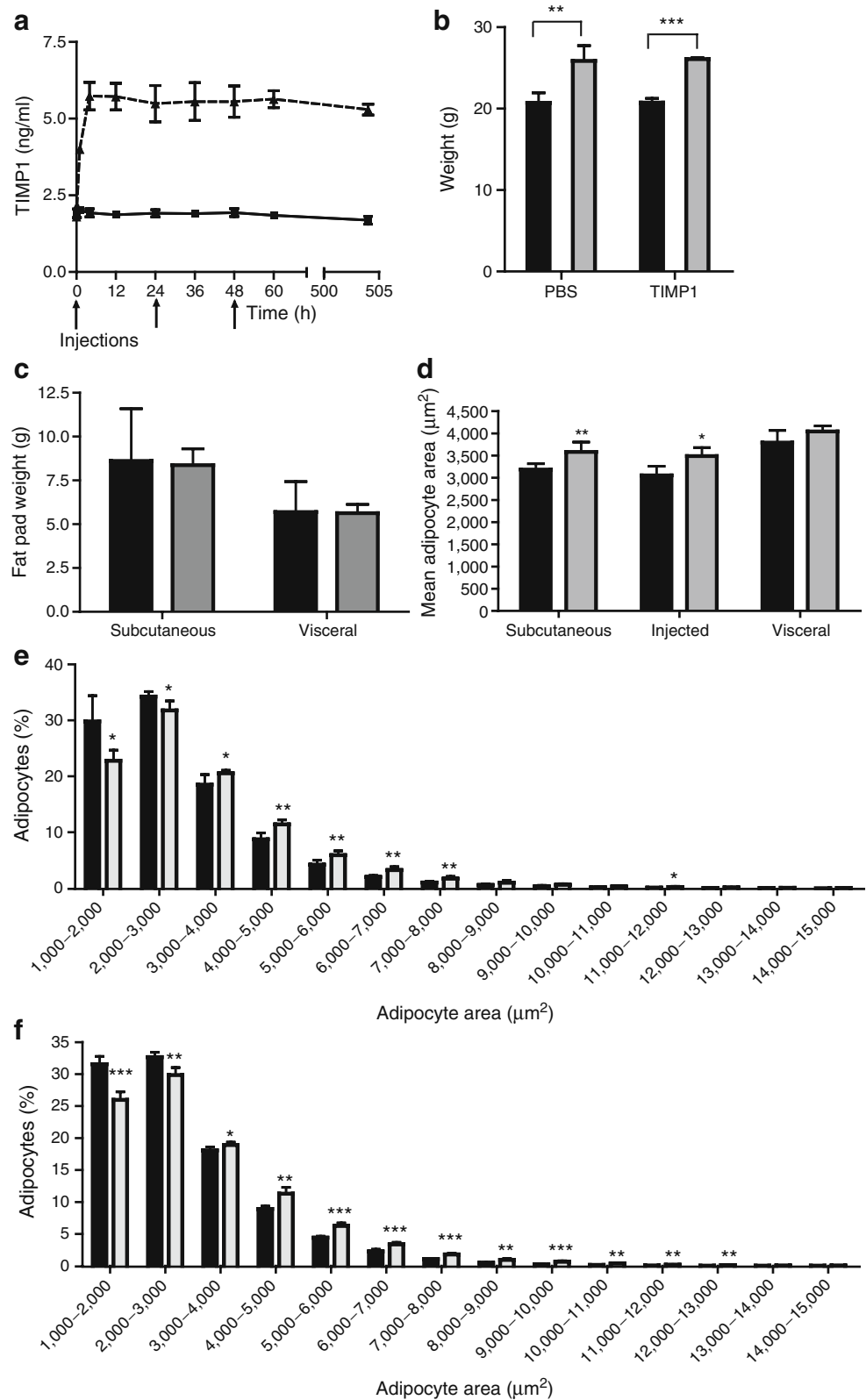
Since it has been reported that some of the observed effects of TIMP1 might be due to a central regulation of energy balance, we tested respiration as well as activity of

**Fig. 3** TIMP1 inhibits primary adipocyte differentiation.

**a** Subcutaneous and visceral SVF from male C57BL/6J mice were differentiated in the presence of different amounts of murine TIMP1 (mTIMP1);  $n=4$ . **b** Representative images of fluorescent staining of differentiated subcutaneous and visceral murine SVF. **c** Oil Red O staining of differentiated subcutaneous SVF in the presence of different amounts of murine TIMP1, as indicated. **d** Subcutaneous and visceral SVF from male C57BL/6J mice were differentiated in the presence of anti-TIMP1 (grey bars) or control antibody (black bars);  $n=4$ ; with **(e)** representative images of fluorescent staining. **f** Inhibition of murine MMP9 (black bars) or human MMP9 (grey bars) by human TIMP1 (hTIMP1). MMP9 activity was measured as fluorescence per min using a fluorescence resonance energy transfer (FRET) substrate peptide;  $n=3$ . **g** Inhibition of murine MMP9 (mMMP9) by different amounts of human TIMP1 (grey bars) or murine TIMP1 (black bars) was measured using a FRET substrate peptide;  $n=3$ . Scale bars (**b**, **c**, **e**) 100  $\mu\text{m}$ . Values (**a**, **d**, **f**, **g**) represent mean  $\pm$  SD; \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$



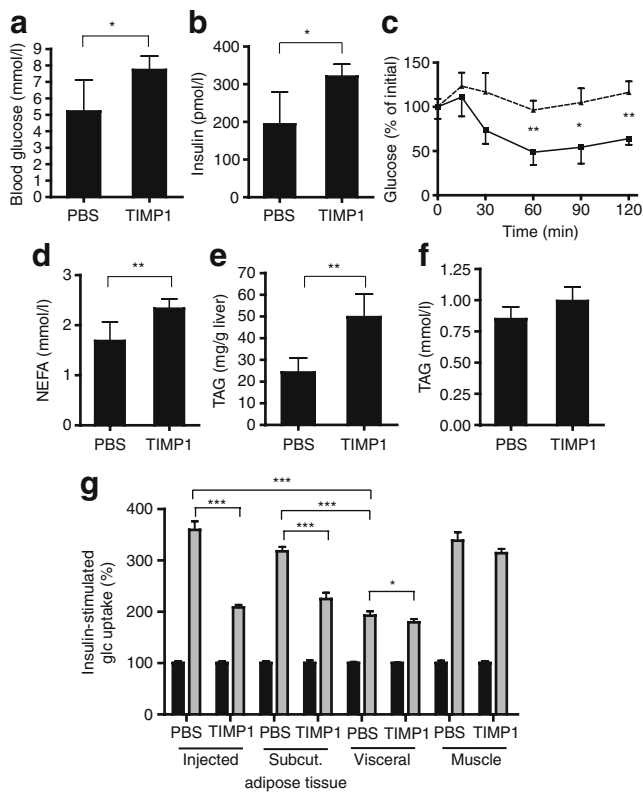
**Fig. 4** Murine TIMP1 leads to adipocyte hypertrophy in vivo. **a** Serum concentration of TIMP1 measured by ELISA during daily injections of murine TIMP1 (triangles) or PBS (squares) into subcutaneous adipose tissue;  $n=5$ . **b** Body weight before (black bars) and after (grey bars) injection of murine TIMP1 or PBS into male C57BL/6J mice in combination with a high-fat diet for 3 weeks;  $n=5$ . **c** Fat pad weight measured by computed tomography of mice injected daily with murine TIMP1 (grey bars) or PBS (black bars), and fed a high-fat diet for 3 weeks;  $n=5$ . **d** Mean adipocyte size of subcutaneous fat at the site of injection, and in ventral subcutaneous and omental fat in male C57BL/6J mice that were injected daily for 3 weeks with murine TIMP1 (grey bars) or PBS (black bars) during high-fat diet;  $n=5$ . **e** Adipocyte size distribution of subcutaneous fat at the site of injection after 3 weeks of murine TIMP (grey bars) or PBS (black bars) injections;  $n=5$ . **f** Adipocyte size distribution of ventral subcutaneous fat (murine TIMP, grey bars; PBS, black bars);  $n=5$ . Values represent mean  $\pm$  SD; \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$



the mice in a metabolism cage system after short-term injections of murine TIMP1 (3 days) and after 3 weeks of murine TIMP1 treatment. Neither short- (data not shown) nor long-term (Fig. 7) murine TIMP1 injections led to

alterations in oxygen consumption, feeding or physical activity, implying that murine TIMP1 injections do not affect regulatory mechanisms of energy homeostasis in the central nervous system.





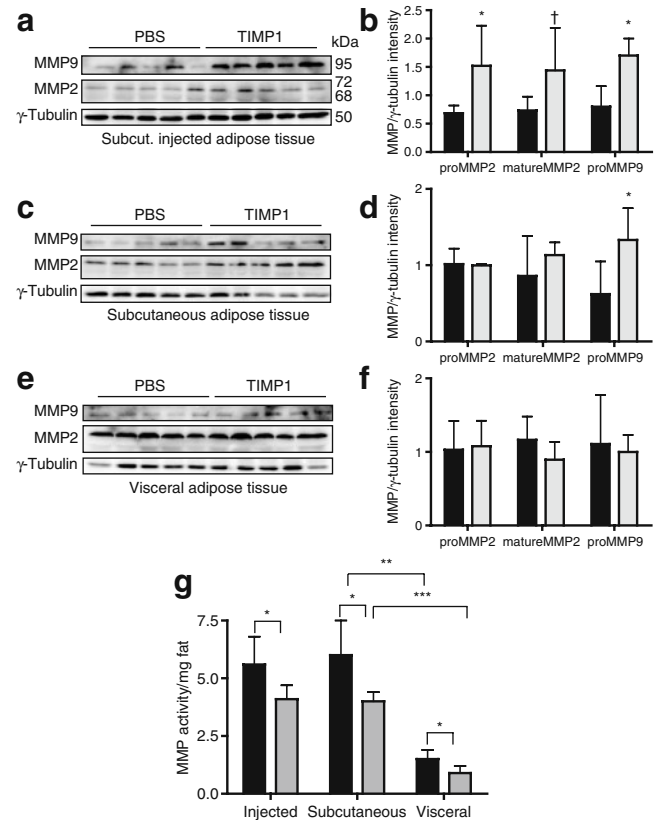
**Fig. 5** Mice injected with murine TIMP1 showed an impaired metabolic profile. **a** Fasting blood glucose and **(b)** insulin serum levels in mice injected with murine TIMP1 or PBS, and on a high-fat diet. **c** Insulin tolerance test of mice injected with murine TIMP1 (triangles) or PBS (squares) and fed a high-fat diet for 3 weeks. Insulin dose: 0.75 U/kg body weight. **a–c**  $n=5$ . **d** Serum NEFA, **(e)** liver triacylglycerol (TAG) content and **(f)** serum triacylglycerol of mice injected with PBS or TIMP1 after 3 weeks of high-fat diet;  $n=5$ . **g** Insulin-stimulated glucose (glc) uptake into adipose tissue and muscle from mice injected with PBS or TIMP1 after 3 weeks of high-fat diet. Basal, black bars; 10 nmol/l insulin, grey bars.  $n=4$ . \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$

In summary, we show here that TIMP1 abundance is upregulated in obesity. TIMP1 inhibits adipocyte differentiation and leads to enlarged adipocytes in vivo. Mice injected with murine TIMP1 mice show impaired NEFA and glucose profiles, increased hepatic triacylglycerol accumulation and faster development of insulin resistance.

## Discussion

Tight regulation of extracellular matrix remodelling by MMPs and their inhibitors such as TIMP1 is crucial for the adipogenic process. The fact that TIMP1 is upregulated in obesity partly due to increased abundance in adipose tissue led to the assumption that TIMP1 influences adipose tissue plasticity in the state of overnutrition.

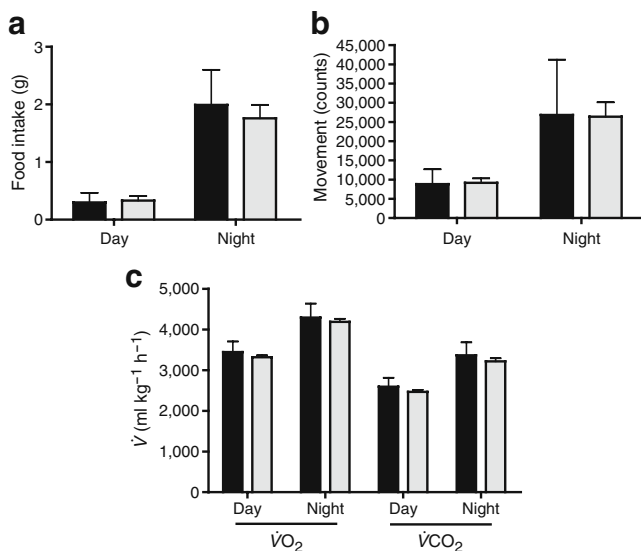
We confirm here that TIMP1 is highly abundant in the SVF of adipose tissue and that levels are higher in visceral



**Fig. 6** **a** Western blot for MMP2, MMP9 and  $\gamma$ -tubulin of protein extracts from subcutaneous injected adipose tissue, with quantification **(b)**; murine TIMP, grey bars; PBS, black bars. **c, d** As above **(a, b)** for subcutaneous adipose tissue and **(e, f)** visceral adipose tissue. **g** Global MMP activity in adipose tissue extracts from mice injected with PBS (black bars) or TIMP1 (grey bars) after 3 weeks of high-fat diet. MMP activity was measured using a fluorescence resonance energy transfer substrate peptide;  $n=4$ . Results are representative of three independent injection experiments with independent protein preparations;  $n=5$ . Values **(b, d, f, g)** are mean  $\pm$  SD; \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , † $p=0.07$

than in subcutaneous adipose tissue [18, 26, 37–39]. In obese animals, TIMP1 secretion from mature adipocytes was increased in all fat depots, whereas TIMP1 secretion from SVF was not altered. These data conflict to some extent with a report from Maury et al. [39], which shows that TIMP1 secretion is higher from SVF and adipocytes in human omental adipose tissue of obese patients compared with lean participants. These differences might be due to species differences or due to different culture and preparation conditions. Based on our observations, it can be assumed that the increase of circulating TIMP1 levels in obesity observed here and by others [26] is at least partly due to increased secretion from mature adipocytes, as TIMP1 levels in other TIMP1-producing tissues are increased to a much lower extent.

As reported previously, TIMP1 production was blunted at induction of adipocyte differentiation, hinting at an inhibitory role of TIMP1 in adipogenesis [18]. We



**Fig. 7** Murine TIMP1 does not affect central energy regulation. **a** Food intake, **(b)** movement and **(c)** respiration in male C57BL/6J mice injected with murine TIMP1 (grey bars) or PBS (black bars) and fed a high-fat diet for 3 weeks;  $n=5$

showed here that murine recombinant TIMP1 repressed adipogenesis in 3T3-L1 pre-adipocytes and in subcutaneous SVF, whereas neutralisation of TIMP1 led to enhanced differentiation. As TIMP1 inhibits a broad spectrum of MMPs, these results are consistent with the fact that global MMP inhibitors repress adipocyte differentiation in vitro [18, 36]. In a previous study, Demeulemeester et al. [29] reported that overabundant human TIMP1 had no effect on adipocyte differentiation in 3T3-F332A pre-adipocytes, whereas Alexander et al. [22] reported enhanced adipogenesis using the MMP inhibitor GM6001 or human TIMP1. The differences between the various reports suggest that the balance between inhibition of different classes of MMPs plays a crucial role. In fact, different classes of MMPs are known to have opposite effects on adipogenesis. Whereas MMP9 and MMP2 are reported to promote adipocyte formation [36, 40, 41], MMP3 or MMP11 inhibit the latter [22, 42]. Additionally, TIMP1 can have MMP-independent effects on cell growth [43]. One future challenge will therefore be to study the ‘mechanics’ of how TIMP1 influences adipogenesis.

In contrast to subcutaneous SVF, no effect of murine TIMP1 or TIMP1 neutralisation could be seen in visceral SVF. It is known that subcutaneous and visceral adipose tissue show distinct gene expression patterns and that visceral fat is a higher risk factor for insulin resistance [11, 44]. As the differentiation capacity of visceral SVF as such was seen here and elsewhere [45] to be much lower, it is likely that other inhibitory factors apart from TIMP1 additionally control adipogenesis in visceral fat.

In vivo, global knockout of *Timp1* leads to sex-specific effects in mice with diet-induced obesity, giving rise to less fat and smaller adipocytes in male animals, whereas female mice with a null mutation develop bigger fat pads and larger adipocytes [27, 28]. One issue is the impact of *Timp1* ablation on the central nervous system, leading to hyperphagia in female mice, which might mask direct effects on adipose tissue development. Male *Timp1* knockout mice do not show altered energy balance, but do display lower body weights as early as at weaning, implying a role for TIMP1 in development. We did not observe any changes in energy balance in murine TIMP1-injected mice, nor did murine TIMP1 injections influence fat pad growth during high-fat diet feeding. This suggests that the observed increase in adipocyte size goes in hand with a decrease in adipocyte number, which is possibly a result of decreased compensatory adipogenesis in mice injected with murine TIMP1.

Demeulemeester et al. reported that chronic overabundance of human TIMP1 had no effect on adipose tissue development [29], whereas Alexander et al. observed more, hypertrophied adipocytes in the mammary gland [22]. Here, we were able to show that injection of murine TIMP1 into subcutaneous fat during 3 weeks of high-fat diet led to enlarged adipocytes in subcutaneous fat pads. Discrepancies can be partially attributed to species-specific differences, as human TIMP1 is less efficient in inhibiting murine MMP9 than human MMP9. Furthermore, human TIMP1 is also less efficient than murine TIMP1 in regulating murine MMP9 activity. In addition, overabundance in the liver [29] might also change activity of human TIMP1 by alternate mechanisms.

Murine TIMP1 injections under a high-fat diet also led to an impaired metabolic profile. It is known that enlarged adipocytes become insulin-resistant and have a high lipolytic rate [8, 9, 46, 47]. As murine TIMP1 injections did not alter muscle glucose uptake, it can be excluded that altered muscle function contributed to the observed changes in glucose metabolism. Although it cannot be ruled out that TIMP1 acts on other metabolic tissues, the elevated serum levels of NEFA seen here hint at a contribution of disturbed adipose tissue function to this phenotype. Elevated NEFA levels in turn cause increased hepatic triacylglycerol accumulation, promoting insulin resistance [4]. Interestingly, a positive correlation was also found between circulating TIMP1 levels and insulin resistance in our group of obese men. This correlation supports the link between TIMP1 levels and insulin resistance.

Murine TIMP1 injections led to decreased MMP activity in adipose tissue, suggesting that the observed metabolic effects are at least partly due to altered adipose tissue MMP activity. Interestingly, MMP activity in visceral vs subcutaneous adipose tissue was much lower in itself, a finding that is consistent with the increased endogenous levels of

TIMP1 in visceral fat. Given the fact that murine TIMP1 inhibited adipogenesis and increased adipocyte size, especially in subcutaneous adipose tissue where endogenous TIMP1 levels are low, it can be speculated that TIMP1 injections increase subcutaneous adipose tissue insulin resistance under high-fat diet feeding, a process that is normally seen predominantly in visceral adipose tissue. This is supported by the fact that insulin-stimulated glucose uptake was lower in visceral than in subcutaneous adipose tissue in control animals and much less affected by TIMP1 treatment.

It is well known that proinflammatory cytokines and adipokines are upregulated in adipose tissue of obese participants and contribute to insulin resistance [48, 49]. In 3T3-L1 adipocytes, TIMP1 can be induced by cytokines such as TNF $\alpha$ , IL-6 and IL-1 $\beta$  [50, 51]. It is therefore intriguing to speculate that increased TIMP1 production in obesity is driven by inflammatory signals, thus inhibiting differentiation of new adipocytes. In fact, it has been shown that the differentiation capacity of SVF from obese mice is impaired, which could in turn lead to adipocyte hypertrophy and increased insulin resistance [52].

Based on these findings, TIMP1 might serve as a target for modulation of adipose tissue plasticity in response to adiposity. Inhibition of TIMP1 would result in an improved hyperplastic response of adipose tissue, which is known to reduce the risk of insulin resistance.

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