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Gelatinase A (MMP-2) promotes murine adipogenesis ☆

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ABSTRACT

Background: Expansion of adipose tissue is dependent on adipogenesis, angiogenesis and extracellular matrix remodeling. A functional role in these processes was suggested for the gelatinase subfamily of the matrix metalloproteinases. Here, we have evaluated a potential role of gelatinase A (MMP-2) in adipogenesis.
Methods: Murine embryonic fibroblasts (MEF) were derived from wild-type or MMP-2 deficient mice. Genetic manipulation of *Mmp2* (shRNA-knockdown or overexpression) was performed in 3T3-F442A preadipocytes. Cell cultures were subjected to an adipogenic medium. As an *in vivo* model for *de novo* adipogenesis, 3T3-F442A preadipocytes with or without knockdown were injected subcutaneously in Nude BALB/c mice kept on high fat diet.
Results: Mmp2 deficient MEF, as compared to controls, showed significantly impaired differentiation into mature adipocytes, as demonstrated by 90% reduced intracellular lipid content and reduced expression of pro-adipogenic markers. Moreover, selective *Mmp2* knockdown in 3T3-F442A preadipocytes resulted in significantly reduced differentiation. In contrast, overexpression of *Mmp2* resulted in markedly enhanced differentiation. In *de novo* formed fat pads resulting from preadipocytes with *Mmp2* knockdown expression of *aP2*, *Ppar-γ* and *adiponectin* was significantly lower, and collagen was more preserved. The fat pad weights as well as size and density of adipocytes or blood vessels were, however, not significantly different from controls.
Conclusion: Our data directly support a functional role of MMP-2 in adipogenesis *in vitro*, and suggest a potential role in *in vivo* adipogenesis.
General significance: Selective modulation of MMP-2 levels affects adipogenesis.

1. Introduction

Obesity is defined as excessive fat accumulation and impairs health. Indeed, obesity is a major contributor to chronic conditions such as cerebro- and cardiovascular diseases and type 2 diabetes [1]. Throughout life, the plasticity of white adipose tissue (AT) remains very high. AT expansion and regression are well-controlled processes in response to the nutritional status. Expansion consists of hypertrophy, where existing adipocytes increase in volume, and hyperplasia, where newly formed adipocytes differentiate from precursor cells (adipogenesis). Tissue growth is also associated with angiogenesis and intensive proteolytic remodeling of the extracellular matrix (ECM) [2,3]. All of these processes are, in part, regulated by the matrix metalloproteinase (MMP) system. The MMP family consists of more than 20 zinc-dependent endoproteinases, which are collectively capable of cleaving nearly all ECM components. The ECM does not only provide a 3D scaffold but also influences cell behavior through interference with several signaling pathways. Furthermore, the organization of the ECM microenvironment is altered during adipogenesis [4,5]. Despite many studies on the MMPs, their individual (patho)physiological functions in AT remain largely unknown. Single gene deficiency of *Mmp9* (92 kDa-gelatinase B), *Mmp10* or *Mmp12* in mice had no apparent effect on adiposity [6–8], whereas deficiency of *Mmp3*, *Mmp11* or *Mmp19* in mice kept on a high fat diet led to enhanced development of AT [9–11]. In contrast, *Mmp14* or *Mmp2* (72 kDa-gelatinase A) deficiency resulted in impaired murine AT development [12,13]. One of the main disadvantages of using MMP-*null* mouse models is that these studies lack information about tissue-specificity. For instance, MMP-2 also plays a role in bone metabolism and *Mmp2 null* mice have a smaller body size [14].

Several studies show that the gelatinase subgroup (MMP-2 and MMP-9) is secreted by AT and that their activity is modulated during AT expansion/regression [15–17]. Elevated plasma levels of MMP-2 are observed in obese patients, and these levels drop after bariatric surgery [18]. Based on *in vitro* studies performed with rat, mouse (3T3-F442A and 3T3-L1 cell lines) and human preadipocytes, a role for the gelatinases has been suggested in differentiation of adipocytes [19–22]. We have previously reported that *Mmp9* gene silencing does not affect differentiation of 3T3-F442A cells [23]. In the present study

☆ *Author sentence* Obesity is a major risk factor for various chronic diseases. Besides surgery, there are no effective treatments available. Understanding the processes involved in adipose tissue expansion is therefore crucial to develop selective therapeutic interventions to target fat tissue/mass.

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we have directly demonstrated the role of MMP-2 in *in vitro* preadipocyte differentiation, and *in vivo de novo* fat pad formation using precursor cells with genetic deficiency, gene silencing or overexpression of MMP-2.

2. Methods

2.1. In vitro cell culture and differentiation

Murine embryonic fibroblasts (MEF) were isolated from 13.5 day old embryos obtained from MMP-2 deficient (*Mmp2*$^{-/-}$) or wild-type (WT) mice. Briefly, embryos were surgically removed and separated from maternal tissues and yolk sac. Heads and intestines were removed, bodies were cut into small pieces and were then incubated in a solution of SMEM and trypsin at 4 °C for 90 min, followed by 12 min at 37 °C. This resulted in a pellet, which was resuspended in basal medium. To initiate differentiation, cells were seeded at a density of 50×10^3 cells cm^{-2} and grown to confluency in basal medium. Medium was refreshed when cells reached confluency. After 48 h, cells were subjected to a two day induction period with DMEM GlutaMAX supplemented with 10% fetal bovine serum (FBS), 1.7 μM insulin, 1 μM dexamethasone (DEX), 0.5 mM methylisobutylxanthine (IBMX) and 5 μM rosiglitazone. Cells were further differentiated in DMEM GlutaMAX containing 10% FBS, 0.85 μM insulin and 5 μM rosiglitazone for 8 days. During the 12-day differentiation period, cell lysates were collected for RNA and protein extraction. Intracellular lipids were stained with Oil Red O to monitor the extent of differentiation. Cells were washed with phosphate-buffered saline (PBS), fixed in 1.5% glutaraldehyde in PBS for 5 min, stained for 3 h with a 0.2% Oil Red O solution (Sigma-Aldrich, Bornem, Belgium), washed and kept in tissue culture water. The stained fat droplets in the cell monolayer were visualized by light microscopy and photographed. For spectrophotometric quantification of lipid accumulation, the Oil Red O dye was extracted with isopropanol and the absorbance of the solution was read at 490 nm on an EL808 plate reader using KC4 DATA ANALYSIS software (Bio-tek Instruments, Winooski, VT, USA).

Murine 3T3-F442A preadipocytes [24] were routinely grown at subconfluence in basal medium (Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Paisley, UK) supplemented with 10% bovine calf serum (BCS, iron supplemented; Hyclone, Logan, UT, USA) and 1% PenStrep (Invitrogen)). Cells were passaged when preconfluent. To initiate differentiation, cells were seeded at a density of 30×10^3 cells cm^{-2} and grown to confluency (designated as 'day 0') in basal medium in an atmosphere of 95% humidified air–5% CO_2 at 37 °C. After 2 days, cells were induced to differentiate for 48 h with induction medium (DMEM supplemented with 10% FBS, 17 nM insulin, 2 nM tri-iodothyronine (T3), 100 nM DEX and 100 mM IBMX). Thereafter, cultures were switched to differentiation medium (DMEM with 10% FBS, 17 nM insulin and 2 nM T3). The medium was replaced with fresh culture medium every 2 days. At regular time points during the differentiation of the cells, cell lysates were taken for RNA extraction. On experimental day 12, the extent of differentiation was assessed by quantification of Oil Red O uptake by lipid-containing cells. Cellular viability was assessed by Trypan Blue dye exclusion assay. At experimental day 12, cells were trypsinized, centrifuged (200 *g* for 5 min) and resuspended in basal medium. Cell suspensions were mixed (1:1) with 0.4% Trypan blue. The number of unviable cells (stained) was counted and expressed as percentages.

2.2. In vitro gene silencing or overexpression

To obtain stable gene silencing of *Mmp2* (NM_008610) in 3T3-F442A preadipocytes, the 'MISSION shRNA lentiviral transduction particles' system (Sigma-Aldrich, St. Louis, MO) was used. Five different target clones were provided (TRCN0000031224–31228) and MISSION non-target shRNA control transduction particles (SHC002V) were used as negative control. The transduction procedure was performed as described elsewhere [25]. Preadipocytes with or without gene silencing were differentiated as described above, except that induction and differentiation medium was enriched with 10% BCS.

The mouse *Mmp2* expression vector (pCMV6-*Mmp2*) and empty plasmid (pCMV6) were obtained from OriGene (Sanbio, Uden, Netherlands). 3T3-F442A cells were transfected using Lipofectamine LTX (ThermoFisher Scientific, Waltham, MA), according to the manufacturer's instructions. Initial selection was done with basal medium containing 5 mg/ml G418. Stable transfectants were maintained in medium containing 2.5 mg/ml G418. Pools of genetically modified cell clones were differentiated as described above.

2.3. De novo adipogenesis model in vivo

The *in vivo* model of *de novo* adipogenesis was established as described elsewhere [25,26]. Briefly, 3T3-F442A preadipocytes (Swiss background) transduced with MMP-2 shRNA construct TRCN0000031228 or SHC002V negative control were injected subcutaneously in the back of 8 week-old male athymic BALB/c Nude mice (purchased from Charles River, Les Oncins, France). Similar experiments were performed in littermate *Mmp2*$^{-/-}$ and WT mice with genetic C57Bl/6 background [13]. Mice were kept on a 12 h day/night cycle for 4 weeks and fed *ad libitum* with a high fat diet (HFD, 21% fat; E15721-34; Ssniff, Soest, Germany). At the end of the experiment, mice were killed by *i.p.* injection of 60 mg kg^{-1} sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL).

Subcutaneous (SC), gonadal (GON), and *de novo* formed fat depots were removed and weighed. Portions were snap-frozen in liquid nitrogen for further analysis. All animal experiments were approved by the local Ethical Committee for Animal Experimentation (KU Leuven, P158/2011) and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996) and EU directive 2010/63/EU.

2.4. Analysis of mRNA and protein levels

Extraction of RNA, subsequent preparation of complementary DNA and quantitative real time PCR on samples from 3T3-F442A cells, MEF or AT were performed as described [27]. The expression of adipocyte fatty acid-binding protein (*aP2*), peroxisome proliferator-activated receptor-γ (*Ppar-γ*, isoform 1 plus 2) and preadipocyte factor 1 (*Pref1*) was determined with the primers and 6-carboxy-fluorescein (FAM) labeled probes reported elsewhere [27]. Expression levels of *Mmp2* (Mm00439498_m1), *Mmp9* (Mm00442991_m1), *Mmp14* (Mm01318969_g1), *Ppar-γ isoform 1 or 2* separately (custom design), Adiponectin (*Adn*; Mm00456425_m1) and Lipoprotein lipase (*Lpl*; Mm00434764_m1) were determined using gene expression assays from Applied Biosystems (ThermoFisher Scientific). Fold differences in gene expression were calculated with the ΔΔCt method, using β-actin as housekeeping gene. β-actin expression did not markedly change (≤10% variation) during the 12-day differentiation period. Control cells at experimental day 0 were used as calibrator.

Commercially available ELISAs were used to determine total antigen levels of MMP-2 (Abcam, Cambridge, UK), MMP-9 (R&D Systems, Oxon, UK) and adiponectin (R&D Systems, Oxon, UK). Gelatinase activity in cultured medium or protein extracts was determined by gel zymography as described [13,28].

2.5. Histology and microscopy analysis

The size and density of adipocytes were determined on paraffin sections of AT, stained with hematoxylin/eosin under standard conditions. Blood vessel staining was performed using the biotinylated Bandeiraea (Griffonia) Simplicifolia BSI lectin (Sigma-Aldrich). Blood vessel density was also normalized to the adipocyte number. Macrophage infiltration was evaluated by staining with an F4/80 antibody (Serotec, Raleigh, NC). Collagen was stained with Sirius Red and quantified as percentage

stained area per total tissue section area. Quality of collagen fibers was estimated by Sirius Red polarization microscopy, allowing to quantify thick, tightly packed collagen fibers (orange–red) and thin, loosely assembled fibers (yellow–green) [29]. Analyses were performed by using a Zeiss Axioplan 2 microscope with AxioVision Rel. 4.8 software (Carl Zeiss, Oberkochen, Germany).

2.6. Statistical analysis

Data are expressed as means ± SEM. Differences between two groups were analyzed with the non-parametric Mann–Whitney *U* test, compatible with small sample sizes. Comparison of progress curves was performed by two-way ANOVA. Bonferroni correction was applied for multiple testing. Analysis was done with Prism 5 (GraphPad Software Inc., San Diego, CA). Values of $p < 0.05$ are considered statistically significant.

3. Results

3.1. *Mmp2*$^{-/-}$ murine embryonic fibroblasts have impaired adipogenic differentiation

Differentiation of MEF derived from *Mmp2*$^{-/-}$ mice was markedly impaired as compared to MEF derived from *Mmp2*$^{+/+}$ mice, as shown by Oil Red O staining after differentiation for 12 days (Fig. 1a). Quantification of the staining confirmed lower intracytoplasmatic lipid content in *Mmp2*$^{-/-}$ MEF (Fig. 1b). This is further supported by significantly reduced expression of the adipogenic marker *aP2* in *Mmp2*$^{-/-}$ cells

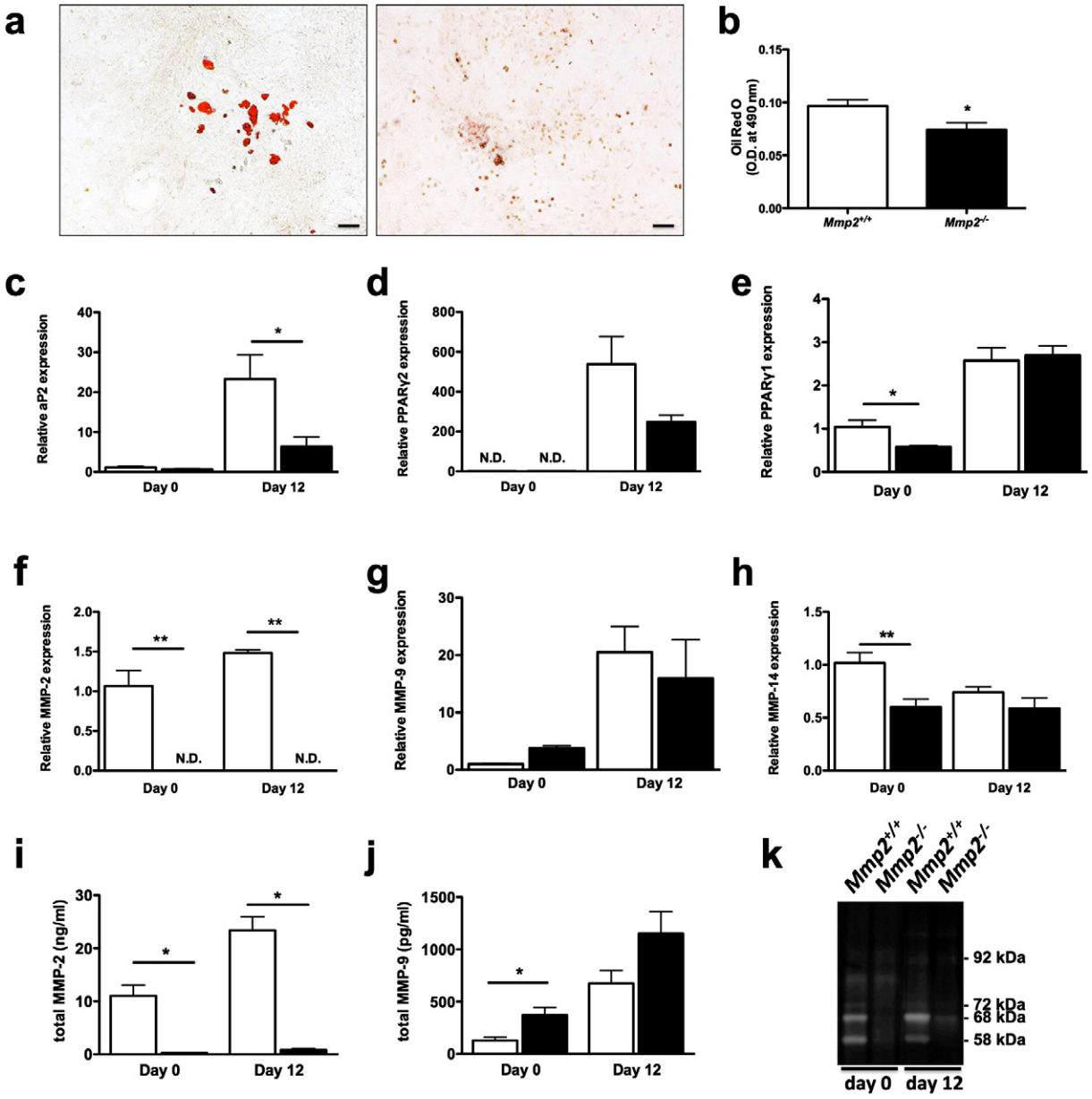

Fig. 1. Differentiation of murine embryonic fibroblasts into mature adipocytes. (a, b) Oil Red O staining (a) and quantification (b) of wild-type (left panel; white bars) and *Mmp2*$^{-/-}$ (right panel; black bars) cells at day 12 of differentiation. The scale bar corresponds to 100 μm. (c–h) Gene expression profile of *aP2* (c), *Ppar-γ2* (d), *Ppar-γ1* (e), *Mmp2* (f), *Mmp9* (g) and *Mmp14* (h) is shown at days 0 and 12. (i, j) Total MMP-2 (i) and MMP-9 (j) antigen levels in conditioned medium at days 0 and 12. (k) Gelatin zymography of conditioned medium. Data are means ± SEM of 3 independent experiments; *$p < 0.05$ and **$p < 0.01$ *versus* WT; N.D., not detected.

after 12 days (Fig. 1c). Expression of *Ppar-γ2* (AT and liver specific isoform) in *Mmp2*$^{-/-}$ MEF showed a trend towards lower levels at day 12 (Fig. 1d), whereas that of *Ppar-γ1* (expressed in many tissues) was lower at day 0 but not at day 12 (Fig. 1e). Determination by ELISA of adiponectin levels in conditioned medium at day 12 revealed lower levels in *Mmp2*$^{-/-}$ MEF (1.9 ± 0.7 ng/ml) in comparison with *Mmp2*$^{+/+}$ MEF (4.7 ± 2.3 ng/ml).

MMP-2 expression at days 0 and 12 was undetectable in *Mmp2*$^{-/-}$ MEF (Fig. 1f), whereas expression of *Mmp9* (Fig. 1g) and *Mmp14* (Fig. 1h) at day 12 was comparable for *Mmp2*$^{-/-}$ and *Mmp2*$^{+/+}$ cells. Total MMP-2 antigen levels in conditioned medium at days 0 and 12 were 11 ± 2.0 ng/ml and 23.4 ± 2.6 ng/ml respectively for *Mmp2*$^{+/+}$ cells and were undetectable for *Mmp2*$^{-/-}$ MEF (Fig. 1i). Total MMP-9 antigen levels in the conditioned medium at day 0 were 127 ± 30 pg/ml *versus* 371 ± 74 pg/ml for *Mmp2*$^{+/+}$ *versus* *Mmp2*$^{-/-}$ cells ($p = 0.01$), with corresponding values of 674 ± 123 pg/ml and 1150 ± 210 pg/ml at day 12 (Fig. 1j). Gelatin zymography with conditioned medium of *Mmp2*$^{-/-}$ cells confirmed the absence of MMP-2 activity at day 0 and 12 (Fig. 1k). In protein extracts (data not shown) of *Mmp2*$^{+/+}$ MEF at days 0 and 12, MMP-2 antigen levels were 10.4 ± 1.9 ng/mg protein and 9.6 ± 0.9 ng/mg protein, respectively, and were undetectable for *Mmp2*$^{-/-}$ MEF. MMP-9 antigen levels in cell extracts at day 0 were 53 ± 15 *versus* 69 ± 9 pg/mg protein for *Mmp2*$^{+/+}$ *versus* *Mmp2*$^{-/-}$ cells, with corresponding values of 114 ± 22 and 156 ± 44 pg/mg protein at day 12. Zymography on gelatin containing gels confirmed the absence of MMP-2 in protein extracts of *Mmp2*$^{-/-}$ in contrast to *Mmp2*$^{+/+}$ MEF; MMP-9 activity was not detectable in the protein extracts (not shown).

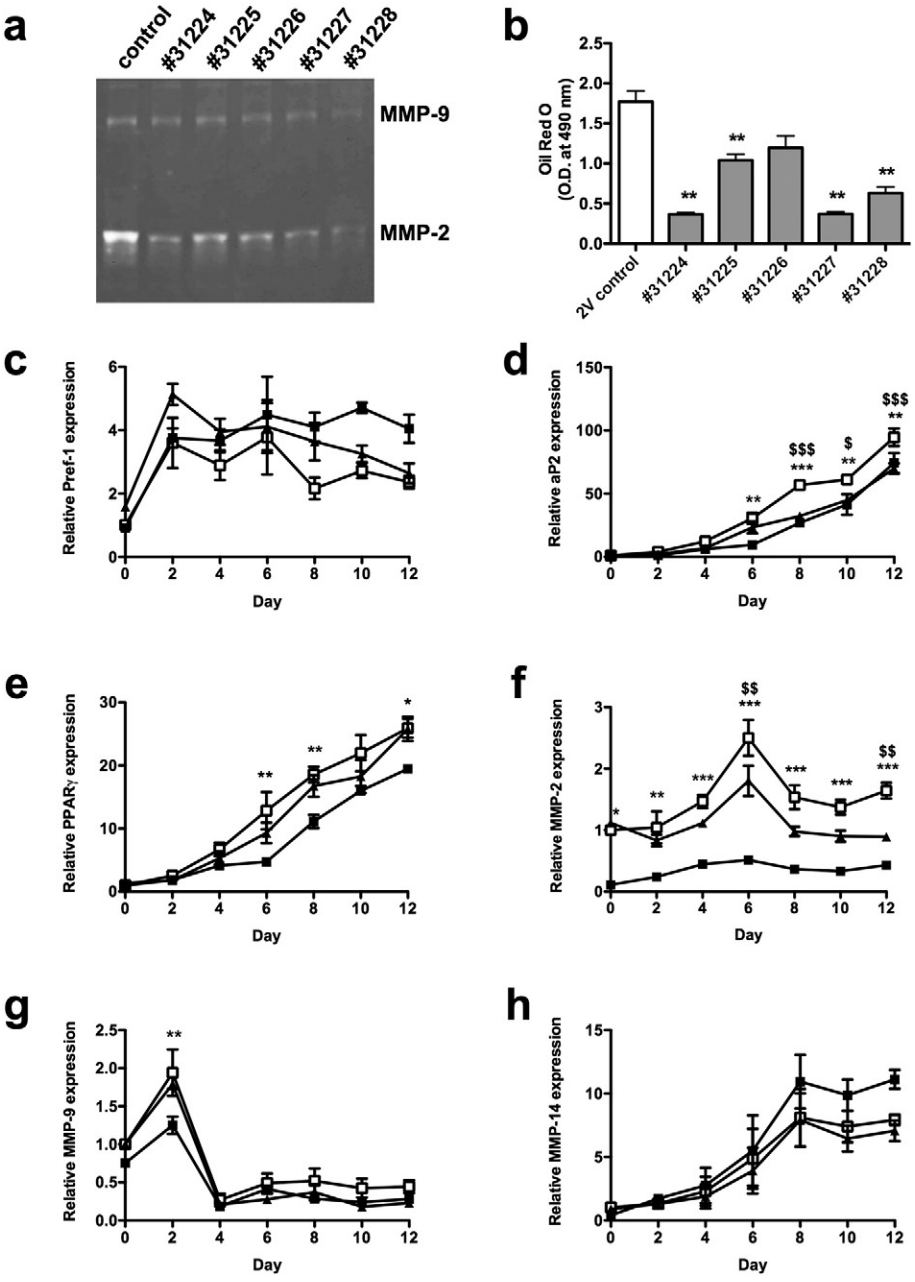

Fig. 2. Effect of *Mmp2* gene silencing on *in vitro* differentiation of 3T3-F442A preadipocytes. (a) Gelatin zymography with conditioned medium of negative control cells (2 V, lane 1) and of cells transduced with 5 different constructs (lanes 2–6) taken at day 0. (b) Quantification of Oil Red O staining at day 12. (c–h) Time course of the expression of *Pref1* (c), *aP2* (d), *Ppar-γ* (e), *Mmp2* (f), *Mmp9* (g) and *Mmp14* (h) during differentiation of 3T3-F442A cells transduced with sh*Mmp2* (black squares), sh*Mmp2′* (triangles) or scrambled shRNA (open squares). Data are means ± SEM of 2 independent experiments performed in triplicate. $^{\$,*}p < 0.05$, $^{\$\$,**}p < 0.01$, and $^{\$\$\$,***}p < 0.001$ *versus* control after Bonferroni correction for multiple testing.

3.2. Mmp2 gene silencing reduces preadipocyte differentiation

Stable *Mmp2* knockdown in 3T3-F442A cells was obtained with all five shRNA lentiviral constructs, ranging between 35 and 92% mRNA downregulation as compared to the SHC002V (hereafter '2 V') control. Additionally, gelatin zymography of conditioned medium (Fig. 2a) confirmed lower levels of MMP-2. Quantification of Oil Red O staining at day 12 revealed reduced differentiation with all constructs ($n = 6$ for each), ranging between 21 and 68% relative to 2 V control (Fig. 2b). Constructs TRCN0000031228 and -31227 (hereafter shMmp2 and shMmp2' respectively) were selected for further analysis. Consequently, monitoring adipogenic mRNA markers during differentiation confirmed higher expression of *Pref1* (Fig. 2c) and significantly lower expression of *aP2* and *Ppar-γ* (Fig. 2d–e), compatible with a lower extent of differentiation upon *Mmp2* knockdown. This is further supported by significantly reduced adiponectin levels in culture medium at day 12 (230 ± 32.1 ng/ml for shMmp2 *versus* 536 ± 12.4 ng/ml for 2 V control; $p = 0.03$).

The time course of mRNA expression indicates that, in contrast to *Mmp2* (Fig. 2f), expression of *Mmp9* and *Mmp14* (Fig. 2g–h) was not impaired during differentiation of 3T3-F442A cells following *Mmp2* knockdown. MMP-2 antigen levels in the medium at day 0 were 1.4 ± 0.03 ng/ml for shMmp2, as compared to 3.4 ± 0.4 ng/ml for control 2 V ($p = 0.03$), with corresponding values of 4.5 ± 0.4 ng/ml and 19.6 ± 1.8 ng/ml at day 12 ($p = 0.03$). MMP-9 antigen levels in the medium were not affected by the knockdown (3.8 ± 0.5 ng/ml for shMmp2 *versus* 3.7 ± 0.6 ng/ml for 2 V at day 0, and 3.1 ± 0.6 ng/ml for shMmp2 *versus* 2.8 ± 0.4 ng/ml for 2 V at day 12). In unconditioned medium, MMP-2 or MMP-9 antigen levels were not detectable.

3.3. Mmp2 overexpression enhances preadipocyte differentiation

Mmp2 overexpression was obtained after transfection of 3T3-F442A cells with an expression vector containing murine *Mmp2* cDNA under control of the CMV promoter. An empty vector (mock) was used as control. At confluence (day 0), *Mmp2* expression was about 50% higher ($p = 0.0006$) in cells with *Mmp2* overexpression, compared to control cells. Elevated levels of total MMP-2 antigen were confirmed in conditioned medium at day 0 (705 ± 62 *versus* 628 ± 72 pg/ml) up to experimental day 12 (20.1 ± 0.4 *versus* 17.6 ± 0.5 ng/ml; $p = 0.002$). Trypan Blue staining at the end of differentiation did not reveal differences in cell death of the 3T3-F442A cells (9.6 ± 0.4 *versus* 8.7 ± 0.8% for control cells). Quantification of Oil Red O staining at days 6, 8 and 12 showed a significant increase in lipid accumulation during differentiation of 3T3-F442A cells with *Mmp2* overexpression (Fig. 3a–b). This was supported by significantly increased expression of *aP2* and *Ppar-γ* from experimental day 6 to 8 (Fig. 3c–d). As from day 8, a plateau in the expression levels was reached for the MMP-2 overexpressing cells. In cells with *Mmp2* overexpression, *Pref1* expression was significantly lower at days 8–10 (Fig. 3e). Adiponectin levels in culture medium at day 8 were higher for MMP-2 overexpressing cells (389 ± 121 *versus* 192 ± 85 ng/ml for controls); this difference was no longer observed at day 12 (923 ± 57.9 *versus* 966 ± 39.4 ng/ml), probably due to the fact that maximal differentiation was reached.

3.4. De novo adipogenesis with Mmp2 gene silencing in BALB/c Nude mice

Transduced 3T3-F442A preadipocytes were used to study adipogenesis *in vivo*. Subcutaneous injection of 3T3-F442A cells, with or without *Mmp2* knockdown (shMmp2 and 2 V respectively), resulted in the formation of *de novo* fat pads in Nude mice after 4 weeks of HFD feeding. Body weight gain was comparable in both groups, resulting in identical body weights and weights of SC and GON AT and *de novo* formed fat pads (Table 1). Histological analysis of sections of *de novo* fat pads revealed no differences in adipocyte size and density (Fig. 4a; Table 1). The size or density of adipocytes in SC and GON AT was also not affected (data not shown). Blood vessel size in *de novo* fat pads was not significantly affected in shMmp2, whereas the density of blood vessels was significantly increased upon *Mmp2* knockdown as compared to controls (Fig. 4a; Table 1). However, this significant increase disappeared after normalization to the adipocyte density.

Macrophage infiltration in the *de novo* fat pads was not significantly different between both conditions (Table 1). Quantification of collagen deposition by Sirius Red staining indicated increased collagen content in the ECM of *de novo* fat pads from shMmp2 cells (Fig. 4a; Table 1). Polarized light analysis showed significantly higher levels of thick collagen fibers (10.0 ± 1.1 *versus* 5.1 ± 0.9%; $p = 0.002$) and lower levels of loosely-organized fibers (28.7 ± 0.6 *versus* 32.3 ± 1.1%; $p = 0.003$) upon *Mmp2* knockdown.

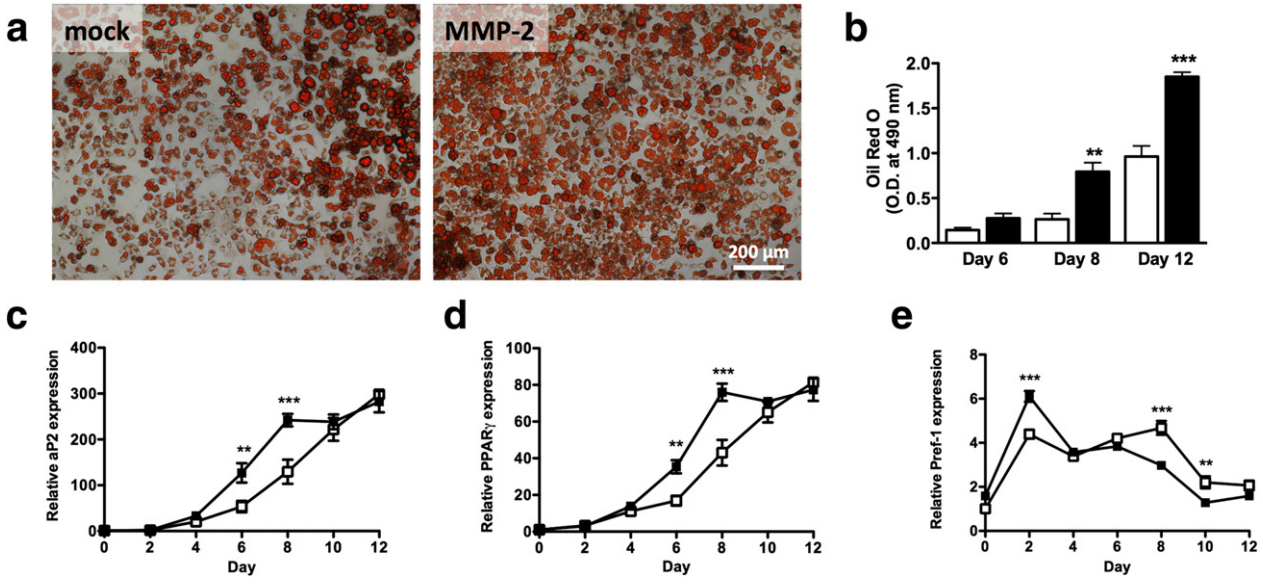

Fig. 3. Effect of *Mmp2* overexpression on *in vitro* differentiation of 3T3-F442A preadipocytes. (a–b) Oil Red O staining at day 12 (a) and quantification at days 6, 8 and 12 of differentiation (b) of cells with (black bars) or without (white bars) *Mmp2* overexpression. (c–e) Time course of mRNA expression of *aP2* (c), *Ppar-γ* (d) and *Pref1* (e) during differentiation of 3T3-F442A cells with (black squares) or without (open squares) *Mmp2* overexpression. Data are means ± SEM of 2 independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control after Bonferroni correction for multiple testing.

Table 1
Body weight and fat depots of Nude mice injected with transduced preadipocytes and kept on high fat diet for 4 weeks.

	Controls ($n = 12$)	shMmp2 ($n = 16$)
Body weight start (g)	18.8 ± 0.3	19.4 ± 0.5
Body weight end (g)	22.6 ± 0.4	23.4 ± 0.4
SC fat (mg)	166 ± 13.4	166 ± 13.3
GON fat (mg)	267 ± 29.9	330 ± 21.9
De novo fat (mg)	28.4 ± 1.1	27.5 ± 1.5
Adipocyte size (μm^2)	674 ± 37.1	703 ± 35.5
Adipocyte density ($\times 10^{-6}/\mu m^2$)	494 ± 40.5	572 ± 30.6
Blood vessel size (μm^2)	24.1 ± 0.8	26.4 ± 1.0
Blood vessel density ($\times 10^{-6}/\mu m^2$)	732 ± 52.0	1005 ± 42.7*
Normalized blood vessel density	1.5 ± 0.1	1.8 ± 0.1
Macrophage infiltration (area %)	1.6 ± 0.4	2.0 ± 0.6
Collagen content	31.3 ± 1.0	36.6 ± 2.2*

Data are means ± SEM. SC, subcutaneous; GON, gonadal.
* $p < 0.05$ *versus* controls.

mRNA analysis in isolated *de novo* fat pads indicated detectable expression of *Mmp2* (albeit approximately 60% lower as compared to control). Expression levels of *Mmp9* and *Mmp14* mRNA were comparable to control cells (Fig. 4b). Determination of total MMP-2 antigen in protein extracts of *de novo* formed fat pads confirmed significantly less MMP-2 for shMmp2 cells (145 ± 43 ng/mg protein) than for controls (331 ± 53 ng/mg protein, $p = 0.03$). This was further confirmed by zymographic analysis, showing markedly lower levels of 68 kDa proMMP-2 and active 58 kDa MMP-2 in protein extracts of shMmp2 *de novo* formed fat pads (Fig. 4c). Total MMP-9 antigen levels were not significantly different in fat pads formed by shMmp2 cells as compared to controls (1.4 ± 0.3 *versus* 2.5 ± 0.6 ng/mg protein). Expression levels of *aP2*, *Adn* and *Ppar-γ1* were significantly lower in *de novo* fat pads with *Mmp2* gene silencing, whereas expression of *Ppar-γ2*, *Lpl* and *Pref1* was not different (Fig. 4d).

Since these data suggest the presence of endogenous MMP-2 in the *de novo* fat pads in Nude mice, the experiment was repeated using

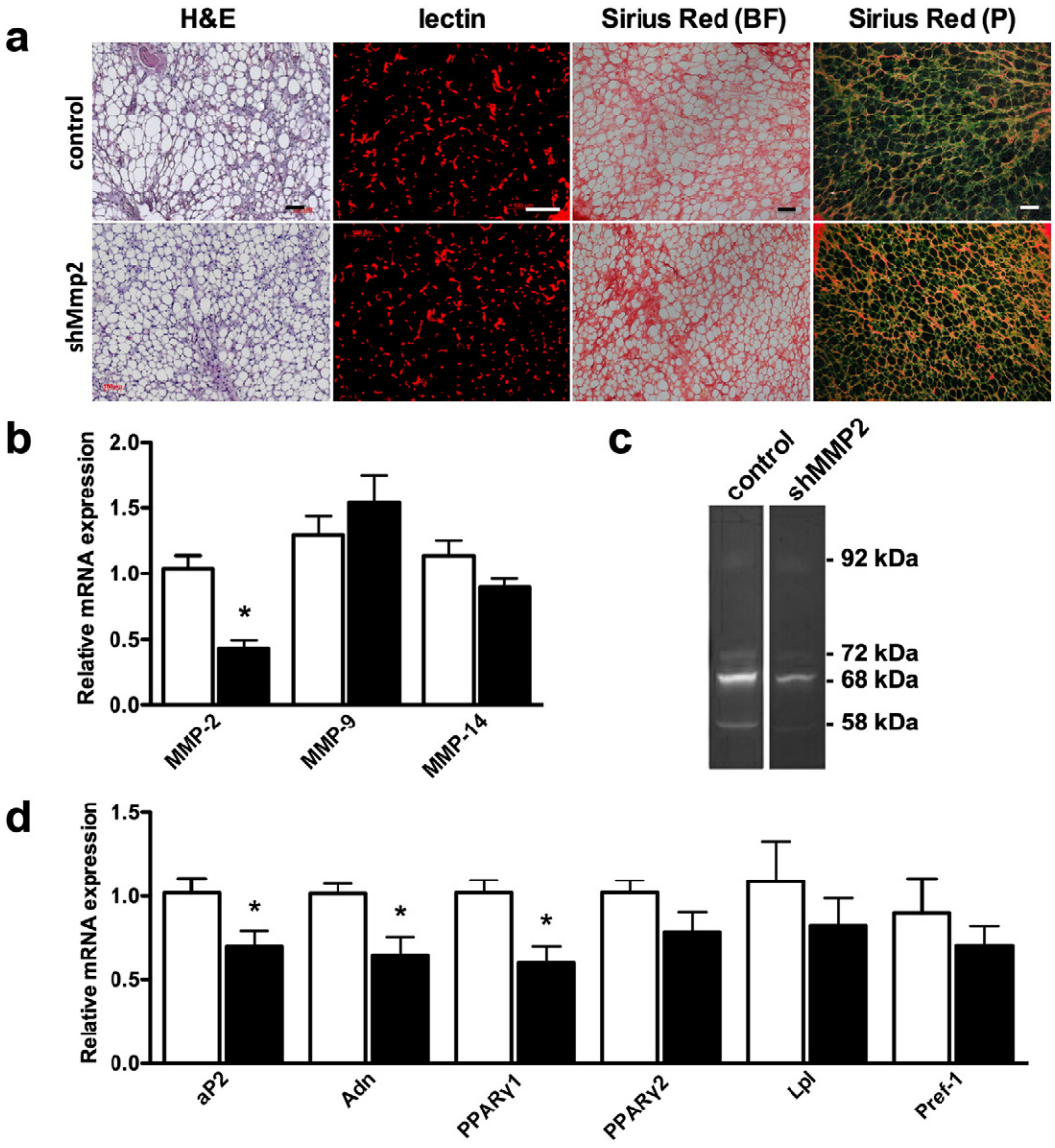

Fig. 4. *De novo* fat pad formation in Nude mice following injection of 3T3-F442A preadipocytes with shMMP2 (black bars) or without (white bars) *Mmp2* gene silencing. (a) Histological stainings with hematoxylin–eosin (H&E), lectin or Sirius Red (BF, bright field; P, polarization) of fat pads derived from 3T3-F442A cells with or without *Mmp2* gene knockdown. Scale bars represent 100 μm. (b) mRNA expression of *Mmp2*, *Mmp9* and *Mmp14*. (c) Gelatin zymography of protein extracts of *de novo* fat pads. (d) mRNA expression of adipogenic markers *aP2*, *Adn*, *Ppar-γ1*, *Ppar-γ2*, *Lpl* and *Pref1*. Data are means ± SEM of 10 experiments; *$p < 0.05$ *versus* control.

Mmp2$^{-/-}$ mice. However, upon injection of 3T3-F442A cells with or without *Mmp2* knockdown in *Mmp2*$^{-/-}$ mice ($n = 8$ for each condition), no *de novo* formed fat pads were detected after 4 weeks of HFD. To exclude a genotype effect, the same experimental protocol was applied with injection of untreated 3T3-F442A cells in WT C57Bl/6*N* mice ($n = 6$), which also failed to result in *de novo* fat pad formation.

4. Discussion

Obesity has become a worldwide problem with ever increasing prevalence rates [30]. A better understanding of AT development and expansion is primordial for better therapeutic intervention. Adipogenesis is an important process in AT biology, for instance to support the approximate 10% renewal of human adipocytes annually [31].

Because of the role MMPs play in AT biology, it has been suggested that modulation of the activity of specific MMPs may affect AT development or growth [32]. Indeed, previous studies have shown the potential to impair AT development in mice by using broad-spectrum MMP inhibitors [33,34]. However, many reports on MMP inhibition contain confounding factors with respect to enzyme specificity, due to cross-inhibition, enzymatic redundancy or compensation. Research over the past years provided evidence for a pivotal role of the gelatinases MMP-2 and -9 [15–17,19–22]. Thus, mice treated with the relative specific gelatinase inhibitor tolylsam had approximately 17% less weight gain on a high fat diet than control mice [13,35].

Although MMP-2 and MMP-9 share overlapping substrates, they are evolved separately, which could explain their differential biological functions [36]. Differences in the response elements of their promotor regions indicate differential gene expression profiles. Interestingly, polymorphisms in the MMP-2 promoter have been associated with obesity in man [37]. Deficiency of *Mmp2*, but not *Mmp9*, in mice kept on high fat diet resulted in impaired AT expansion [7,13]. Likewise, *Mmp14* deficiency in young mice resulted in diminished AT development [12]. MMP-14 is known to play an important role in proMMP-2 activation.

In agreement with the *in vivo* study on MMP-9, we have previously demonstrated that stable gene silencing of *Mmp9* does not affect differentiation of 3T3-F442A preadipocytes [7,23]. In the present study, we have directly evaluated the role of MMP-2 in different stages of adipogenesis. Differentiation of preadipocytes into new mature adipocytes can be divided in two main phases [38]. First, the determination phase consisting of commitment of progenitor cells to the adipocyte lineage. Secondly, during terminal differentiation, preadipocytes acquire the specific characteristics of mature adipocytes. The murine 3T3-F442A preadipocytes are a committed unipotent cell line, eligible to study *in vitro* and *in vivo* preadipocyte differentiation [24].

Here, we show that by reducing the levels of MMP-2 via selective gene silencing, the differentiation of 3T3-F442A cells is reduced compared to control cells. This was monitored by Oil Red O staining, mRNA expression of adipogenic markers and adiponectin levels in culture medium. Levels of MMP-2 remained low throughout the differentiation period and other relevant MMPs such as MMP-9 and MMP-14 did not compensate for the *Mmp2* knockdown. As expected, *Mmp2* overexpression had the opposed effect and stimulated differentiation of 3T3-F442A preadipocytes. It should be noted, however, that MMP-2 overexpression is only modest. A selection bias of adipogenic clones may thereby not be excluded. However, even with minimally enhanced MMP-2 levels, enhanced differentiation was supported by Oil Red O staining and expression analysis of adipogenic markers.

Moreover, MEF derived from *Mmp2*$^{-/-}$ mice and subjected to an adipogenic hormonal cocktail, showed impaired differentiation into mature adipocytes, as compared to *Mmp2*$^{+/+}$ MEF. We observed that the overall degree of MEF differentiation was relatively low, which could be contributed to the genetic background (C57Bl/6N) [39].

Thus, MMP-2 appears to play a functional role in early and later stages of adipogenesis *in vitro*. These findings are in line with diet studies with transgenic mice that indicate that MMP-2, but not MMP-9, is an important player in AT development. Surprisingly, previous studies with 3T3-F442A cells treated with the MMP inhibitors tolylsam or ABT-518 showed increased differentiation, possibly due to lack of specificity [27,40]. Therefore, our present studies with specific inactivation/overexpression or genetic deficiency of MMP-2 in precursor cells more conclusively establish a functional role for MMP-2.

To extend our *in vitro* findings, we subcutaneously injected the 3T3-F442A cells with *Mmp2* knockdown in Nude mice. This is an established *in vivo* model of *de novo* adipogenesis [26], for which it was shown that cytoplasmic triglyceride-containing vesicles reached maximal size at 4–6 weeks. Gene expression analysis of *de novo* formed fat pads revealed lower levels of adipogenic markers for *Mmp2* knockdown cells. However, the newly formed fat pads derived from cells with *Mmp2* knockdown did not differ from the controls with respect to weight or to size or density of adipocytes and blood vessels. Since macrophages infiltrate in obese AT [41] and have been shown to communicate with preadipocytes to influence how they differentiate and survive [42], we have determined the macrophage content of the *de novo* formed fat pads. There was, however, no effect of *Mmp2* gene silencing in the injected preadipocytes on macrophage content of the *de novo* fat pads.

We cannot exclude contribution of endogenous MMP-2 in *de novo* fat pad formation, as MMP-2 antigen and activity were detected in extracts of *de novo* fat pads developed from preadipocytes with gene silencing (albeit at significantly reduced levels, as compared to controls). Actually, endothelial cells produce and secrete MMPs and it has been reported that *in vivo* adipogenesis is dependent on angiogenesis [43]. Thus, local levels of active MMP-2 (independent of its origin) may be sufficient to account for its role in adipogenesis. Therefore, we attempted to perform this experiment in *Mmp2*$^{-/-}$ mice ($n = 16$; C57Bl/6N background) and subsequently in WT mice ($n = 6$). Unfortunately, these additional experiments suggested that ectopic fat pad formation is not supported in this genetic background, possibly due to efficient clearance by the host immune system. A small additional pilot study was performed, with WT mice treated with cyclosporine (Neoral; 40 mg kg^{-1} day^{-1} by oral gavage) to prevent rejection of the injected preadipocytes. This resulted in the formation of tissue enriched in mature adipocytes, but not with the characteristic adipose morphology. Therefore, the role of MMP-2 in *in vivo* adipogenesis was not further explored in this model.

Soukas *et al.* compared expression profiles of 3T3-L1 *in vitro* cultured cells with peri-uterine fat of female C57Bl/6J and *ob*/*ob* mice [44], and concluded that the *in vitro* and *in vivo* transcriptional profiles are, although somewhat overlapping, yet very distinct. This suggests that extrapolation from *in vitro* observations on adipogenesis to the *in vivo* situation should be done with great care. It is known that the ECM composition/rigidity affects adipocyte differentiation [38]. MMP-2 degrades for instance collagen type IV, the major component of basement membranes. Lower MMP-2 activity levels could thus result in preservation of structurally intact collagen, which may hamper the hypertrophic growth of adipocytes [33]. A potential shortcoming of currently used *in vitro* systems is that they consist of a simple cell monolayer. More in depth investigation of mechanisms of adipogenesis may require the use of 3D cell culture systems. However, culture plates coated with either gelatin, collagen type I or type IV did not affect the outcome of the *in vitro* experiments (data not shown). In addition, there is a potential effect of intracellular substrate cleavage by MMP-2 [45], as MMP-14 may induce intracellular proMMP-2 activation. A potential effect of these MMP-2 actions on adipogenesis has not been reported.

In conclusion, we directly demonstrate a role for MMP-2 in different stages of *in vitro* adipogenesis, whereas direct evidence for its role in *in vivo* adipogenesis remains elusive, but would seem compatible with the observation that *Mmp2* deficiency in mice is associated with impaired AT development in a model of nutritionally-induced obesity [13].

Conflicts of interest statement

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

All authors participated in the research design. D.B., I.S. and M.VH. carried out the experiments, collected data and DB performed data analysis. D.B., M.VH. and R.L. performed data interpretation. All authors were involved in writing the paper and had approval of the final version.

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