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Inhibition of PPAR γ , adipogenesis and insulin sensitivity by MAGED1

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Abstract

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a master regulator of adipogenesis and a target of the thiazolidinedione (TZD) class of antidiabetic drugs; therefore, identifying novel regulators of PPAR γ action in adipocytes is essential for the future development of therapeutics for diabetes. MAGE family member D1 (MAGED1), by acting as an adaptor for ubiquitin-dependent degradation pathways and a co-factor for transcription, plays an important role in neural development, cell differentiation and circadian rhythm. Here, we showed that MAGED1 expression was downregulated during adipogenesis and loss of MAGED1 promoted preadipocyte proliferation and differentiation *in vitro*. MAGED1 bound to PPAR γ and suppressed the stability and transcriptional activity of PPAR γ . Compared to WT littermates, MAGED1-deficient mice showed increased levels of PPAR γ protein and its target genes, more CD29⁺CD34⁺Sca-1⁺ adipocyte precursors and hyperplasia of white adipose tissues (WATs). Moreover, MAGED1-deficient mice developed late-onset obesity as a result of decreased energy expenditure and physical activity. However, these mice were metabolically healthy as shown by improved glucose clearance and insulin sensitivity, normal levels of serum lipids and enhanced secretion of adipokines such as leptin and adiponectin. Taken together, our data identify MAGED1 as a novel negative regulator of PPAR γ activity, adipogenesis and insulin sensitivity in mice. MAGED1 might therefore serve as a novel pharmaceutical target to treat obesity-associated insulin resistance.

Key Words

- ▶ obesity
- ▶ insulin sensitivity
- ▶ PPAR γ stability
- ▶ energy expenditure
- ▶ MAGE gene family

Journal of Endocrinology
(2018) **239**, 167–180

Introduction

The obesity epidemic continues to rise as a global health challenge. Obesity is a major risk factor for high blood pressure, hyperlipidemia, diabetes, heart disease, cancers, etc. (Ng *et al.* 2014, Ogden *et al.* 2014). The white adipose tissue (WAT) serves as a critical integrator of energy

balance and glucose homeostasis by storing excess energy to prevent ectopic fat accumulation in non-adipose tissues and by secreting various adipokines to modulate whole-body metabolism (Ouchi *et al.* 2011). Adipocyte hyperplasia, or adipogenesis, is the process of adipocyte

differentiation from pre-adipocytes, which reside in the stromal vascular fraction (SVF) of WAT (Rosen & MacDougald 2006, Gesta *et al.* 2007, Tran & Kahn 2010). The nuclear receptor peroxisome proliferator-activated receptor- γ (PPAR γ) is a master regulator of adipogenesis and most pro- and anti-adipogenic factors seem to function at least in part by regulating PPAR γ expression and/or activity (Farmer 2006, Rosen & MacDougald 2006). The thiazolidinedione (TZD) class of antidiabetic drugs agonize PPAR γ to activate adipogenesis and sensitize insulin action (Spiegelman 1998). The PPAR γ protein is short-lived as it can be ubiquitinated and degraded through the proteasome (Floyd & Stephens 2002, van Beekum *et al.* 2009, Kim *et al.* 2014). Activation by TZDs further accelerates PPAR γ degradation; therefore, understanding how PPAR γ stability is controlled will shed light on new strategies to fine-tune this metabolic master regulator to treat type 2 diabetes.

Melanoma antigen (MAGE) family member D1 (MAGED1), also known as neurotrophin receptor-interacting MAGE (NRAGE) and DLXIN-1, plays an important role in transcription, cell signaling and protein stability (Di Certo *et al.* 2007, Doyle *et al.* 2010, Wang *et al.* 2010, Mouri *et al.* 2012). Growing evidence demonstrates that MAGED1 interacts with proteins such as DLX5 (Masuda *et al.* 2001), p75NTR (Salehi *et al.* 2000), ITA and XIAP (Jordan *et al.* 2001), UNC5H1 (Williams *et al.* 2003), ROR2 (Matsuda *et al.* 2003) and ROR α (Wang *et al.* 2010) to regulate neural development, cell apoptosis and proliferation and circadian rhythm. We and others have showed that MAGED1-knockout mice exhibit a shortened circadian period and bouts (Wang *et al.* 2010), impaired learning and memory (Yang *et al.* 2015) and an osteoporotic phenotype (Liu *et al.* 2015). However, the role of MAGED1 in energy and glucose metabolism has not been studied.

Recently, MAGE family proteins were shown to bind really interesting new gene (RING) domain proteins to form active E3 ubiquitin ligases (Doyle *et al.* 2010). For example, the MAGEC2-TRIM28 complex targets p53 for proteasomal degradation (Doyle *et al.* 2010). The MAGEL2-TRIM27 promotes K63-linked ubiquitination of WASH, thus facilitating endosomal F-actin nucleation and retromer-mediated transport (Hao *et al.* 2013). Likewise, MAGED1 interacts with RING proteins including PRAJA-1 (Sasaki *et al.* 2002), and TRIM28 to a less extent (Doyle *et al.* 2010). In addition, MAGED1 controls the degradation of the anti-apoptotic factor CHE-1 (Di Certo *et al.* 2007), and the activity and ubiquitylation of the serotonin transporter (Mouri *et al.* 2012). In this study, we report that MAGED1 interacts with PPAR γ and decreases its stability. Loss of

MAGED1 leads to enhanced preadipocyte proliferation and adipogenesis. MAGED1-deficient mice develop obesity but lack metabolic abnormalities including dyslipidemia, impaired glucose tolerance and insulin resistance.

Materials and methods

Animals

C57BL/6J mice from Jackson Laboratory and *Maged1*-knockout (KO) mice (Wang *et al.* 2010) were maintained in an AAALAC-accredited, specific pathogen-free facility in Model Animal Research Center of Nanjing University. All animal protocols were approved by Institutional Animal Care and Use Committee of Nanjing University, in accordance with published guidelines in the Principles of Laboratory Animal and the Care and Use of Laboratory Animals (National Research Council 1996). All mice were maintained under 12-h light/darkness cycles (light on at 08:00h and off at 20:00h) and fed with acidified water and standard chow *ad libitum*. *Maged1*-KO mice had been backcrossed to C57BL/6J mice for at least 15 generations.

Serum biochemistry parameters measurements

Mice were fasted for overnight or 6h or refed for 2h as indicated. Whole blood was collected from the eye socket vein. Serum was collected after a centrifugation at 3000g for 15 min. Lipid metabolites in plasma including total cholesterol, triglyceride (TG), high-density lipoprotein and low-density lipoprotein (VLDL) were quantified by colorimetric assays with a 7020 automatic analyzer (Hatachi High Technology, Japan).

Glucose and insulin tolerance tests

For oral glucose tolerance test, mice were fasted from 17:00 to 09:00h and injected with D(+)-glucose (Sigma, 2g/kg body weight). For insulin tolerance test (ITT), mice were fasted from 09:00 to 15:00h and injected with 0.75 U insulin (Novo Nordisk) per 1 kg body weight. Blood glucose concentrations were determined by GLUCOCARD II Test Meters (Arkray, Minneapolis, MN, USA).

Metabolic characterization and body composition analyses

The Comprehensive Lab Animal Monitoring System was used to determine food intake, physical activity, oxygen

consumption and carbon dioxide production. All mice were maintained in a 12:12 light/darkness cycle at 22°C and acclimated for 2 days. Data were recorded for at least 72 h for analyses.

A dual-energy X-ray absorptiometry system (GE Medical System Lunar, Madison, WI, USA) was used to determine body composition. Mice were anesthetized with Avertin (Sigma Aldrich) and kept on a heat plate for data acquisition.

Histology

Tissues were collected and fixed in 4% formalin (in PBS) overnight, embedded in paraffin and sectioned at a thickness of 5 µm. Slides were dewaxed with xylene, rehydrated with descending grades of ethanol and then rinsed with distilled water. Tissue sections were then stained with hematoxylin and eosin. ImageJ was used for quantification of adipocyte size and area from histological slides. In brief, images were transformed into 8-bit and the image threshold was set to selectively visualize adipocyte prior analyses. All the images were acquired with the Coolscope Microscope (Nikon).

For immunohistochemistry, tissue sections were deparaffinized, antigen was retrieved by steaming for 30 min and then cooled to room temperature. Slides were blocked with 5% BSA for 1 h, incubated with anti-BrdU primary antibody (rat monoclonal, Abcam) overnight at 4°C and secondary antibody conjugated with cyanine 5 (eBioscience). Images were observed with a confocal laser-scanning microscope (FV1000, Olympus MicroImaging).

Isolation and differentiation of adipose SVF cells

Adipose tissues were excised, washed with PBS and minced in digestion buffer (100 mM HEPES pH 7.4, 120 mM NaCl, 50 mM KCl, 5 mM glucose, 1 mM CaCl₂, 1.5% BSA, and 1 mg/mL collagenase D (Roche)). Then, the mixture was digested at 37°C for 30 min with shaking at 85 rpm. The slurry was filtered through 40 µm cell strainer to remove undigested bulks and large adipocytes. After that the aqueous phase was centrifuged for 5 min at 500g, the pellet was resuspended in preadipocytes culture medium (DMEM/F12 with Glutamine, 10 mM HEPES, and 25 µg/mL sodium ascorbate). Preadipocytes were seeded on 6 cm dishes, and medium was changed next morning. Cells were passaged at confluence and frozen for further use.

For adipocyte differentiation, preadipocytes were cultured on poly-lysine-coated dishes to confluence in preadipocyte medium. Cells were kept at confluence for another 24 h to arrest the cell cycle into the G1 phase. Cells were then induced by basic differentiation medium

containing the adipogenic cocktail (850 nM Insulin, 0.5 µM dexamethasone, 250 µM isobutylmethylxanthine (IBMX) and 1 µM rosiglitazone) for 48 h. Cells were then kept in basic preadipocyte culture medium with 850 nM insulin till analyses.

To make Oil-Red O stock, 500 mg of Oil-Red O was dissolved in 100 mL of isopropanol and kept stirring overnight, avoiding of light. The solution was filtered and stored in dark cabinet for further use. Adipocytes were fixed with 4% formalin for 1 h, washed with 1× PBS three times and stained with the Oil-Red O solution for 1 h. Cells washed with deionized water for four times were imaged. Finally, 1 mL isopropanol was added to dried wells to dissolve Oil-Red O for the colorimetric detection.

Flow cytometry

WATs were excised, washed in PBS solution twice and then minced in the digestion buffer (100 mM HEPES pH 7.4, 120 mM NaCl, 50 mM KCl, 5 mM glucose, 1 mM CaCl₂, 1.5% BSA, and 1 mg/mL collagenase D (Roche)). Tissues were transferred to 15 mL tubes and incubated at 37°C with vibrations at 87 rpm. Cell pellets were obtained by centrifugation at 500g for 5 min and resuspended in 1 mL PBS. After filtering through 100 µm strainers, cells were fixed in 70% ethanol for 20 min and incubated with 0.1% TritonX-100 in 5% BSA solution for 1 h. Cells were stained with antibodies against Sca-1-PE, CD29-PE-Cy5 and CD34-APC (BD Biosciences) on ice for 1 h. Samples were analyzed with a FACSCalibur flow cytometer and the CellQuest software (BD Biosciences).

For MTT assay, passage two preadipocytes were seeded at a density of 8×10^3 per well in 96-well plates. A total of 20 µL of 5 mg/mL MTT were added to each well after 48 h of culture. Cells were incubated at 37°C for 4 h. Culture medium was removed carefully, 150 µL MTT solvent was added and plates were shaken at 60 rpm for 15 min avoiding of light. The absorbance was read at 590 nm with a reference filter of 620 nm.

Mouse embryonic fibroblasts preparation and culture

Embryos at day 12.5 were dissected, washed in PBS and transferred into drops of digestion solution (0.25% trypsin, 0.04% EDTA). Tissues were minced and incubated in 3 mL of digestion solution at 37°C for 15 min. Three milliliters of DMEM plus 10% FBS were added and then incubated at 37°C with occasional shaking for another 15 min. Cells were pelleted by centrifugation at 300g for 5 min and resuspended in DMEM containing 10% FBS for the culture.

SV40T antigen integrated lentivirus was used to infect the P2 mouse embryonic fibroblasts (MEFs). Puromycin-selected cells were considered as immortalized MEFs.

Cell culture, transient transfection and luciferase assay

HEK293, NIH/3T3 and 3T3-L1 cells were cultured in DMEM medium supplemented with 10% FBS at 37°C, 5% CO₂. 3T3-L1 cells were differentiated similarly as primary SVFs. Briefly, overconfluent 3T3-L1 cells were induced with 1 µg/mL Insulin, 1 µM dexamethasone, 0.5 mM IBMX and 1 µM troglitazone for 2 days, and then maintained with 1 µg/mL insulin and 1 µM troglitazone for another 6 days. Cells were harvested at indicated days for RNA and protein extraction.

Cells were transiently transfected with equal molar amounts of luciferase-fusion PPAR γ 1 and PPAR γ 2 constructs or pGL3-basic backbone in antibiotics-free growth media using the Lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen). pRL-tK (Promega) containing the Renilla luciferase gene was co-transfected in order to normalize transfection efficiencies. To examine the ability of MAGED1 in regulation the transcription of *Pparg*, pCDNA3.1-MAGED1 vector, fragments those containing the proximal promoters of *Pparg1* (–1500bp) and *Pparg2* (–677, –2275bp) incorporated into the luciferase-fusion reporter constructs, and pRL-tK were transfected. Cells were then lysed at 48 h after transfection, and the luciferase activities were determined using the dual-luciferase reporter assay system (Promega).

Quantitative RT-PCR

Total RNA from adipocyte tissues was extracted with RNAiso plus (9108, TaKaRa). Primary cDNA was synthesized by the PrimeScript RT Kit together with the gDNA Eraser kit (RR047A, TaKaRa). Quantitative RT-PCR was performed using the SYBR Premix Ex Taq (RR420A, TaKaRa) in an ABI 7700 sequence detector (Applied Biosystems). The relative abundance of target gene transcripts was normalized to levels of *36B4*.

Protein isolation, immunoprecipitation and Western blot

Cells were washed and lysed in denaturing lysis buffer (30mM HEPES at pH 7.6, 100mM NaCl, 0.5% Nonidet P-40, protease inhibitor cocktail from Roche, 1mM Na₃VO₄ and 20mM NaF) on ice for 10 min and centrifuged

at 12,000g for 10 min. Tissues were collected quickly after killing mice by cervical dislocation, dipped into liquid nitrogen and homogenized immediately in complete RIPA lysis buffer (50mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA and protease inhibitors) on ice-water bath. The homogenate was incubated at 4°C for 1 h with continuous rotation. Protein concentration was measured by Bradford's method (Protein assay dye reagent concentrate, Cat. No. 500-0006, Bio-Rad).

Five hundred micrograms of protein samples were incubated with 1 µg of indicated antibodies and protein A/G beads on a rotator at 4°C overnight. The bead-antibody-antigen complexes were washed four times in PBS with protein inhibitors. The beads were finally boiled in 80 µL 1× loading buffer and the supernatant was collected for further analyses (Cao et al. 2010, Svensson et al. 2013).

Protein samples were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% fat-free milk in TBST (0.1% Tween-20 in TBS) and incubated with antibodies diluted in 5% BSA/TBST at 4°C overnight. Next day, membranes were washed with TBST for 4×5 min, incubated with HRP-conjugated secondary antibody diluted in 5% fat-free milk/TBST at room temperature for at least 1 h, washed with TBS and detected using ECL Western Blotting substrates (Cat. No. 32106, Pierce). If needed, membranes were stripped with stripping buffer (2% SDS, 62.5 mM Tris, pH 6.8, 100mM 2-mercaptoethanol) at 50°C for 30 min.

Statistical analyses

Data were presented as the means ± S.E.M. Statistical differences were determined by unpaired Student's *t*-test (for two groups) or one-way ANOVA with Tukey's multiple comparisons test (for three or more groups).

Results

MAGED1 negatively regulates adipogenesis

To determine the potential role of MAGED1 in adipogenesis, we first examined its expression during the differentiation of 3T3-L1 preadipocytes. We found that levels of both *Maged1* mRNA and MAGED1 protein gradually decreased after the adipogenic induction (Fig. 1A and B). In the gonadal WATs (gWAT) in mice, the expression of *Maged1* mRNA reduced when adipogenesis

was induced by high-fat diet (HFD) feeding (Fig. 1C). These data indicate that MAGED1 expression is negatively associated with adipogenesis. To directly determine the effect of MAGED1 on adipogenesis, we differentiated WT and MAGED1-KO MEFs into the adipogenic lineage with IBMX, dexamethasone and insulin (collectively termed as MDI) or MDI plus triiodothyronine (T3). MAGED1-deficient MEFs showed increased efficiency in adipogenesis when compared to WT MEFs (Fig. 1D). Consistent with this result, the adipogenic response of SVF cells from gWAT was more robust in KO mice than in WT mice (Fig. 1E). We found that the expression of marker genes, such as total *Pparg*, *Pparg2* and *Adipoq*, was significantly upregulated in induced KO adipocytes, particularly at late time points (Fig. 1F, G and H). The nuclear receptor PPAR γ is a master regulator of adipogenesis (Farmer 2006, Rosen & MacDougald 2006), and levels of PPAR γ protein were evidently increased in induced KO adipocytes, when compared to WT controls (Fig. 1I). Collectively, these data demonstrate that MAGED1 is a negative regulator of adipogenesis.

MAGED1 inhibits the stability and activity of PPAR γ

In its MAGE domain, MAGED1 contains two LXXLL motifs that are required for interactions with nuclear receptors

(Supplementary Fig. 1A, see section on supplementary data given at the end of this article). Indeed, immunoprecipitation showed that MAGED1 bound to both PPAR γ 1 and PPAR γ 2 (Fig. 2A). We then assayed protein stability using cycloheximide (CHX), which is an inhibitor of protein biosynthesis (Ruan et al. 2012). MAGED1 overexpression reduced the stability of both PPAR γ 1 and PPAR γ 2 (Fig. 2B and C). In addition, using a PPAR response element (PPRE)-driven luciferase reporter, we found that MAGED1 prevented the induction of luciferase activity by PPAR γ 1 in 3T3-L1 cells (Fig. 2D) and NIH/3T3 cells (Supplementary Fig. 1B). PPAR γ binds to its own promoter and activates its own transcription (Lefterova et al. 2008, Nielsen et al. 2008). We also observed decreased *Pparg1* and *Pparg2* promoter activities when MAGED1 was overexpressed in 3T3-L1 cells (Fig. 2E) and NIH/3T3 cells (Supplementary Fig. 1C, D, E and F). On the other hand, MAGED1 knockdown in NIH/3T3 cells increased *Pparg2* promoter activities (Supplementary Fig. 1G). These data suggest that MAGED1 may inhibit adipogenesis by suppressing the stability and activity of PPAR γ .

Increased adiposity in MAGED1-KO mice

We then sought to determine whether MAGED1 regulated adipogenesis *in vivo*. In young male mice when there was

Figure 1

MAGED1 controls adipogenesis *in vitro*. (A) Expression levels of *Maged1* during the adipogenic induction of 3T3-L1 cells ($n=6$). (B) Expression of MAGED1 and PPAR γ proteins during 3T3-L1 adipocyte differentiation. (C) mRNA levels of *Maged1* in gWAT from mice fed with NC or HFD ($n=4-5$). (D) WT and KO MEFs were differentiated into adipocytes by MDI or MDI plus T3 and stained with Oil-Red O. The staining was quantified by spectrophotometry at OD 500 of the dye extracted from cells ($n=3$). (E) SVF cells from gWAT of WT and KO mice were differentiated into adipocytes and Oil-Red O staining was quantified ($n=9$). (F, G and H) Relative mRNA levels of total *Pparg*, *Pparg2* and *Adipoq* in differentiated WT and KO SVFs ($n=6-7$). (I) Expression of PPAR γ protein in differentiated WT and KO SVFs. Data presented as mean $\pm$ s.e.m. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ by one-way ANOVA with Tukey's multiple comparisons test (A) and two-tailed *t*-test (C, D, E, F, G and H).

Figure 2

MAGED1 inhibits PPAR γ stability and activity. (A) FLAG-tagged MAGED1 was co-expressed with MYC-tagged PPAR γ 1 or PPAR γ 2 in HEK 293 cells and their interactions were determined by reciprocal immunoprecipitation with anti-MYC and anti-FLAG antibodies. Normal IgG was used for immunoprecipitation in the last lane as negative controls. (B and C) PPAR γ 1 (B) and PPAR γ 2 (C) were co-transfected with vector or MAGED1 in HEK 293 cells and then treated with CHX for indicated times. Expression of PPAR γ was determined by immunoblotting. Relative levels of PPAR γ protein were shown below the Myc blots. (D) PPRE-luciferase assay in 3T3-L1 cells ($n=4$). (E) *Pparg*1 and *Pparg*2 promoter-driver luciferase assay in the absence/presence of MAGED1 in 3T3-L1 cells ($n=4$). Data presented as mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ by One-way ANOVA with Tukey's multiple comparisons test (D) and two-tailed t-test (E).

no difference in body weight (Fig. 3A), no difference in WAT weight was observed at 2 weeks (Fig. 3B). At 3 weeks of age, there was a trending increase in weight of gonadal and posterior subcutaneous WAT in KO mice (Fig. 3C). At 4 weeks of age, compared to WT mice, KO mice had significantly heavier gonadal, posterior subcutaneous and interscapular WAT and showed a tendency to increase in inguinal and perirenal fat weight (Fig. 3D). No change in brown adipose tissue (BAT) weight was observed (Fig. 3B, C and D), indicating that the effect of MAGED1 on adipogenesis is either specific to WAT only or not evident in BAT at this developmental stage. We then further analyzed gWAT and found no changes in adipocyte number or size at 2 weeks (Fig. 3E, F and G). We could observe adipocyte hyperplasia starting at 3 weeks of age (Fig. 3E) and adipocyte hypertrophy not until 4 weeks of age (Fig. 3F, G and H). These data demonstrate that loss of MAGED1 in mice results in increased adiposity in early life.

Consistent with the inhibitory effect of MAGED1 on PPAR γ *in vitro*, we found that PPAR γ protein levels were substantially upregulated in gWAT in KO mice (Fig. 4A).

mRNA levels of *Pparg* and its target genes involved in adipogenesis, lipogenesis and glucose metabolism were all increased in KO mice (Fig. 4B). These results demonstrate that loss of MAGED1 increases PPAR γ levels and promotes adipogenesis *in vivo*.

Cell proliferation is essential for adipocyte differentiation (Fajas 2003). The MTT assay showed that SVF cells from KO mice had higher proliferation rates than WT cells *in vitro* (Fig. 4C). Regulators of cell cycle progression, including Cyclin D1 and A2, increased their expression in KO SVF cells (Fig. 4D). To directly assess adipocyte proliferation *in vivo*, we performed bromodeoxyuridine (BrdU) labeling in mice from postnatal day 7 to 14. Immunostaining in gWAT showed that significantly more BrdU-tracked adipocytes were present in KO mice than WT mice (Fig. 4E and F). We then performed flow cytometry of isolated SVF cells from gWAT and found KO mice had more CD29⁺CD34⁺Sca-1⁺ adipocyte precursors (Fig. 4G) (Rodeheffer *et al.* 2008, Berry & Rodeheffer 2013), suggesting an increased adipogenic potential in MAGED1-deficient mice.

Figure 3

Increased adiposity in young MAGED1-KO mice. (A) Body weight of 2–4 weeks old WT and KO mice ($n=7-13$). (B, C and D) Weight of different fat depots in 2-week-old (B, $n=7-13$), 3-week-old (C, $n=6-11$) and 4-week-old (D, $n=5-16$) mice. (E and F) Adipocyte numbers (E) and cell sizes (F) in gWAT ($n=20$). (G) Representative H&E images of gWAT. (H) Frequency distribution of adipocyte sizes in gWAT ($n=20$). Data presented as mean $\pm$ s.e.m. or scatter dot blot with lines at mean and s.e.m. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ by two-tailed t -test.

Figure 4

Enhancement of adipogenesis in MAGED1 KO mice. (A) Expression of PPAR γ in gWAT shown by Western blotting. (B) Quantitative RT-PCR of PPAR γ -target genes in gWAT in 4-week-old mice ($n=5$). (C) MTT assay of cultured preadipocytes ($n=11-14$). (D) Expression of Cyclin D1 and A2 in culture SVF cells. (E) Immunostaining of BrdU in gWAT from mice injected with BrdU from postnatal day 7 to 13. (F) Quantification of the percentage of BrdU+ adipocytes ($n=7$). (G) Frequency of CD29+CD34+Sca-1+ adipocyte precursors in gonadal SVF cells ($n=3$). Data presented as mean $\pm$ s.e.m. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ by two-tailed t -test.

MAGED1-KO mice develop late-onset obesity

We then sought to determine the long-term energy homeostasis in MAGED1-KO mice. We found that KO males gained more weight than WT mice starting from around week 19 (Fig. 5A). Body composition analyses showed that there was no difference in lean mass and only fat weight and percentage were increased in KO mice (Fig. 5B). Consistent with findings in young mice (Fig. 3), all WAT depots were hypertrophic (Fig. 5C). Similarly, KO females also developed late-onset obesity (Fig. 5D), suggesting no sex difference in the effect of MAGED1 on body weight control. We then profiled serum levels of adipokines and lipids. Similar to increased mRNA levels of *Lep* and *Adipoq* in KO adipose tissue (Fig. 4), levels of serum LEPTIN (Fig. 5E and F) and ADIPONECTIN (Fig. 5G and H) in KO mice were higher than WT mice, even before obesity was apparent. However, we did not observe any changes in serum levels of triglyceride, cholesterol or free fatty acids at all ages we examined (Fig. 5I, J and

K), indicating that increased fat was mostly stored in adipocytes and did not induce lipotoxicity in KO mice.

To determine what caused obesity in KO mice, we performed metabolic cage studies. At 6 weeks of age when no difference in body weight was observed, KO male mice consumed less food at some time points, but total food intake was comparable between WT and KO mice (Fig. 6A). A tendency of reduced RER in KO mice was observed (Fig. 6B). Total energy expenditure indicated by calculated heat production was reduced in KO mice during early hours into the dark phase (Fig. 6C). Reduced physical activity was also observed in KO mice, particularly during the dark phase (Fig. 6D). We then repeated the metabolic cage study in 4-month-old male mice and body weight-matched mice were selected to avoid confounding factors in calculating energy metabolism (Tschop *et al.* 2012). We found that 4-month-old KO mice consumed less food during the dark phase (Fig. 6E). RER was consistently lower in KO mice during the dark phase and the early light phase (Fig. 6F), as a result of reduced food intake and/or

Figure 5

Late-onset obesity in MAGED1-deficient mice. (A) Growth curve of male WT and KO mice ($n=9$). (B) Body composition of 7 months old male WT and KO mice ($n=9$). (C) Adipose weight of 1-year-old male WT and KO mice ($n=4-5$). (D) Growth curve of female WT and KO mice ($n=10-11$). (E and F) Serum levels of leptin in fed (E) and fasted (F) mice ($n=3-6$). (G and H) Serum levels of adiponectin in fed (G, $n=6-9$) and fasted (H, $n=6-7$) mice. (I, J and K) Serum levels of triglyceride (I), cholesterol (J) and free fatty acid (K) ($n=5-10$). Data presented as mean $\pm$ s.e.m. or scatter dot blot with lines at mean and s.e.m. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ by two-tailed *t*-test.

Figure 6

Energy metabolism in MAGED1 mice. (A, B, C and D) Metabolic cage studies of 6-week-old male WT and KO mice ($n=7-9$) showing food intake (A), RER (B), heat production (C) and physical activity (D). (E, F, G and H) Metabolic cage studies of body weight-matched 4-month-old male WT and KO mice ($n=8$) showing food intake (E), RER (F), heat production (G) and physical activity (H). Absolute levels during the day and night shown at the top and average levels shown at the bottom. Data presented as mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ by two-tailed t -test.

preferred fat utilization. Heat production (Fig. 6G) and physical activity (Fig. 6H) were consistently lower in KO mice during the dark phase. However, we did not observe any changes in the morphology (Supplementary Fig. 2A) or UCP1 expression (Supplementary Fig. 2B) in BAT in KO mice. Taken together, these data indicate that MAGED1 KO mice develop energy imbalance and late-onset obesity.

Improved glucose metabolism and insulin sensitivity in MAGED1 KO mice

Activation of PPAR γ in adipose tissues improves glucose tolerance and insulin sensitivity in diabetic patients and animal models (Spiegelman 1998). We then assessed systemic glucose metabolism in MAGED1-KO mice. Glucose levels in KO mice were significantly lower than WT mice during glucose tolerance tests at ages of 3 months (Fig. 7A) and 6 months (Fig. 7B). ITTs demonstrated that KO mice had improved insulin sensitivity compared to WT mice before and after their body adiposity was increased (Fig. 7C and D). The reduction in fasting glucose concentrations in KO mice was apparent at 3 and 6 months but lost when mice were more than 1 year old (Fig. 7E and F). However, these old KO mice still

had lower fasting insulin levels (Fig. 7G) and reduced scores of the homeostatic model assessment of insulin resistance (HOMA-IR, Fig. 7H). Similar levels of fasting serum glucagon suggested that the improved glucose metabolism was not a result of functional changes in pancreatic α cells (Fig. 7I). Hepatic glucose production was not affected in KO mice, shown by the pyruvate tolerance test (Supplementary Fig. 3A) and gluconeogenic gene expression (Supplementary Fig. 3B). Glucose-stimulated insulin secretion *in vivo* (Supplementary Fig. 3C) and in isolated pancreatic islets (Supplementary Fig. 3D) was also normal, indicating intact β cell function in KO mice. These data demonstrate that obesity in MAGED1-KO mice was not associated with an impairment in glucose metabolism, possibly because enhanced adipogenesis and lipid-storing ability eliminated the deleterious effects of lipids on tissues like liver and pancreas.

Finally, we assessed insulin signaling directly. Treating MEFs with increasing concentrations of insulin for 5 min (Fig. 8A) or 100 nM insulin for various time points (Fig. 8B) showed that AKT phosphorylation and total IRS1 levels were upregulated while phosphorylated JNK was decreased in KO cells, indicating that loss of MAGED1 sensitizes insulin signaling. Consistently,

Figure 7

Glucose metabolism in MAGED1 mice. (A and B) Glucose tolerance tests with a dose of 2 g/kg BW glucose in 3 months (A, $n=11-13$) and 6-month-old (B, $n=5-7$) mice. Area under curves shown to the right. (C and D) Insulin tolerance tests with a dose of 0.75 U/kg BW insulin in 3-month (C, $n=12-14$) and 6-month (D, $n=7$)-old mice. Area under curves shown to the right. (E) Blood glucose levels of overnight-fasted mice at different ages ($n=4-13$). (F, G, H and I) 15.5 months old mice were fasted overnight and levels of blood glucose (F), serum insulin (G), HOMA-IR (H) and serum glucagon (I) were determined ($n=4-5$). Data presented as mean $\pm$ S.E.M. or scatter dot blot with lines at mean and S.E.M. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ by two-tailed t -test.

phosphorylation of AKT at S473 was increased in gWAT of KO mice, compared to WT controls (Fig. 8C). Levels of JNK phosphorylation, however, were comparable between genotypes (Fig. 8C). Obesity generally is associated with a state of chronic, low-grade inflammation (Xu *et al.* 2003). However, the expression of inflammatory genes including *Il6* and *Tnfa* was not changed in gWAT in KO mice (Fig. 8D). Similarly, serum levels of TNF α were not elevated in KO mice at all ages examined (Fig. 8E). Taken together, our data demonstrate that loss of MAGED1 in mice enhances insulin sensitivity and improves glucose metabolism.

Discussion

MAGE family consists of more than 50 genes, most of which are localized in clusters in the X chromosome (Chomez *et al.* 2001, Lee & Potts 2017). They can be

broadly categorized into the cancer-testis antigen type I that includes MAGE-A, -B and -C, and the ubiquitous type II that includes MAGE-D, -E, -F, -G, -H, -L and NECDIN. While type I MAGEs control germ cell development and function as oncogenes to drive tumorigenesis, type II MAGEs are important for neurodevelopment (Lee & Potts 2017). *MAGEL2* and *NECDIN* are two of the five genes inactivated in Prader-Willi syndrome (PWS), a genetic condition that causes hyperphagia and severe obesity in affected children. Mice lacking the *Magel2* gene phenocopy human PWS, being overweight with increased adiposity (Bischof *et al.* 2007, Mercer *et al.* 2013). Disruption of the *Necdin* gene in mice also results in hypothalamic and behavioral defects reminiscent of human PWS (Muscatelli *et al.* 2000). NECDIN is highly expressed in preadipocytes and loss of NECDIN promotes adipogenesis *in vitro* and adipose hyperplasia *in vivo* (Bush & Wevrick 2012, Fujiwara *et al.* 2012). Here, we showed that MAGED1 is another MAGE family member that negatively regulates

Figure 8

MAGED1 controls insulin sensitivity. (A and B) Immortalized WT and KO MEFs were treated with various concentrations of insulin for 5 min (A) or 100 nM insulin for various times (B). Total proteins were run for immunoblotting with indicated antibodies. (C) gWAT proteins from *ad libitum*-fed 8-month-old WT and KO mice were subjected to immunoblotting ($n=5$). (D) Expression of *Il6* and *Tnfa* genes in gWAT ($n=5$). (E) Serum levels of TNF α in WT and KO mice at different ages ($n=3-8$). Data presented as mean $\pm$ s.e.m. or scatter dot blot with lines at mean and s.e.m.

adipogenesis and MAGED1 deficiency leads to adipose hyperplasia and obesity in mice. It will be interesting to determine in the future whether other type II MAGEs also modulate adipogenesis and systemic metabolism and whether a unified mechanism can be adopted.

Besides promoting adipogenesis, MAGED1-KO mice also show reduced food intake and physical activity when compared to WT littermates, suggesting possible functional defects in the hypothalamus. In supporting its neuronal function, MAGED1 was previously shown to regulate neuronal differentiation and survival (Salehi *et al.* 2000), modulate circadian rhythm (Wang *et al.* 2010) and be involved in depression (Mouri *et al.* 2012), learning and memory formation (Yang *et al.* 2015), and social interactions and sexual behavior (Dombret *et al.* 2012). Of note, Dombret *et al.* also observed that MAGED1-KO mice develop very-late-onset obesity (when 1 year old) that is associated with hyperphagia and reduced motor activity (Dombret *et al.* 2012). However, obesity could be seen in our MAGED1-KO mice much earlier in both sexes (Fig. 5) and increased adipogenesis could be observed as early as 1 month old. In addition, food intake is reduced in our MAGED1-KO mice (Fig. 6). These discrepancies between the two studies could be a result of different knockout strategies used. Dombret *et al.* deleted exons

4–12 of the *Maged1* gene and a truncated MAGED1 fragment translated from the remaining exons 2–3 could be detected. We replaced exons 3 through 8 with the PGK-Neo cassette, and we could not detect any *Maged1* mRNA or residual MAGED1 protein (data not shown). Nevertheless, future investigations using tissue-specific *Maged1* deletions would help delineate the potential role of MAGED1 in hypothalamic control of metabolism.

Elegant biochemical and biophysical studies by the Potts group discovered that MAGE proteins bind to and activate the E3 RING ubiquitin ligases (Doyle *et al.* 2010, Hao *et al.* 2013, Lee & Potts 2017). They found that MAGED1 forms a strong complex with PRAJA-1 and a weak complex with TRIM28 (Doyle *et al.* 2010). A previous study using yeast two-hybrid screening also identified PRAJA-1 as a MAGED1-interacting protein (Sasaki *et al.* 2002). The E3 ligase activity of PRAJA-1 causes a decrease in MAGED1 protein level, thus controlling the transcription function of the homeodomain protein DLX5. Here, we demonstrated that MAGED1 interacts with both PPAR γ 1 and PPAR γ 2. Overexpression of MAGED1 reduces the stability and transcriptional activity of PPAR γ 1 and PPAR γ 2. Various E3 ligases have been reported to control PPAR γ protein levels, including NEDD4 (Li *et al.* 2016) and RING proteins including ARIH2, c-CBL, LRSAM-1, RNF4, SIAH2,

MKRN1 and TRIM23 (Kilroy et al. 2012, Kim et al. 2014, Watanabe et al. 2015). Future experiments are warranted to determine whether and which RING proteins mediate the interaction and ubiquitination of PPAR γ proteins by MAGED1.

In MEF cells where PPAR γ is not expressed, we found that MAGED1 KO increased IRS1 expression and insulin signaling activity (Fig. 8). IRS1, a critical mediator of the insulin pathway, has been extensively shown to be regulated by ubiquitination-dependent degradation (Xu et al. 2008, Song et al. 2013, Yang et al. 2016). We suspect that MAGED1 may function as an adaptor protein for E3 ligases to target IRS1 for degradation. Therefore, in MAGED1-KO mice, increased IRS1 expression and insulin signaling can also function upstream to activate PPAR γ and promote adipogenesis.

Taken together, MAGED1 negatively regulates PPAR γ stability and activity, adipogenesis and insulin sensitivity. Harnessing MAGED1 may represent a novel approach for the treatment of obesity, diabetes and related metabolic diseases.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0349>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by National Natural and Science Foundation of China (31500944) to Z H; Ministry of Science and Technology of China (2014BAI02B01 and 2015BAI08B02) and National Natural Science Foundation of China (31772550) to X G; and American Heart Association (14SDG20120052), American Diabetes Association (18-IBS-167) and National Natural and Science Foundation of China (81770543) to H-B R.

Author contribution statement

Q W and J T performed most of the experiments and data analyses with the help from S J, Z H, A S and S H. Q W wrote the manuscript, together with J T. X G supervised the project and contributed to manuscript editing. H B R conceived the project, performed experiments, analyzed data and wrote the manuscript.

Acknowledgments

The authors thank Xiaoyong Yang (Yale University) for providing pGL3-Pparg1(–1500bp)-luc, pGL3-Pparg1(–160bp)-luc and pGL3-Pparg2(–1000bp)-luc plasmids.

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Received in final form 19 July 2018

Accepted 14 August 2018

Accepted Preprint published online 18 August 2018