Novel hormone-regulated genes in visceral adipose tissue: cloning and identification of proinflammatory cytokine-like mouse and human *MEDA-7*: implications for obesity, insulin resistance and the metabolic syndrome

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Abstract

Aims/hypothesis We sought to characterise novel genes dysregulated by sex hormonal imbalances that induce obesity and metabolic disorder in a setting of oestrogen deficiency and androgen dominance in follicle-stimulating hormone receptor (*For* [also known as *Fshr*]) knockout female mice.

Methods Transcriptome analysis of mesenteric adipose tissue (MAT) of mutants revealed novel genes. One novel gene named *Meda-7* was selected for study. *Meda-7* was cloned from mouse and human adipose tissue; its expression, hormonal regulation and function were characterised.

Results Mouse *Meda-7* is richly expressed in deep visceral adipose tissue and encodes a 22 kDa secreted protein with

71% homology to human mesenteric oestrogen-dependent adipose gene- 7 (*MEDA-7*) protein. Both have six conserved cysteines like many cytokines. In obese patients, *MEDA-7* is more abundant in omental than subcutaneous fat. *Meda-7* is downregulated in *For*-knockout female MAT at 5 months (obese state) followed by steep upregulation at 9 months (prediabetic condition) when mutants progress towards the metabolic syndrome. *Meda-7* is expressed predominantly in the stromal–vascular cell fraction. In this fraction, M1-proinflammatory macrophages are rich in *Meda-7*. *Meda-7* dysregulation in 5-month-old *For*-knock-out MAT is restored by oestrogen, but treatment has no effect in older mutants. Overabundance of *MEDA-7* in HEK-293 cells enhances cell proliferation via p42/44 mitogen-activated protein kinases. Secreted *MEDA-7* attenuates insulin-stimulated glucose uptake in 3T3-L1 adipocytes, while downregulating glucose transporter-4 and upregulating both monocyte chemotactic protein-1 and suppressor of cytokine signalling-3. Downstream activity of the insulin signalling mediator, phospho-AKT, is also downregulated. **Conclusions/interpretation** *MEDA-7* is a hormone-regulated adipokine/proinflammatory cytokine that is implicated in causing chronic inflammation, affecting cellular expansion and blunting insulin response in adipocytes.

M.R. Sairam dedicates this article to the memory of his mentor Emeritus Professor Dr N.R. Moudgal.

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Abbreviations

CCL	Chemokine (C-C motif) ligand
EST	Expressed sequence tag
FSH	Follicle-stimulating hormone
GFP	Green fluorescent protein
HFD	High-fat diet

LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAT	Mesenteric adipose tissue
MCP	Monocyte chemotactic protein
MEDA-7	Mesenteric oestrogen-dependent adipose gene 7
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ORF	Open reading frame
PACAP	Proapoptotic caspase-2- and caspase-9-binding protein
PAT	Perigonadal adipose tissue
SAT	Subcutaneous adipose tissue
SVC	Stromal vascular cell
VAT	Visceral adipose tissue
WAT	White adipose tissue

Introduction

Visceral obesity as a major risk factor for the metabolic syndrome is increased in postmenopausal women [1] and in sex hormone imbalance endocrine disorders such as hyperandrogenaemia in polycystic ovary syndrome [2]. These women have a higher risk of cardiovascular disease and type 2 diabetes [3–5]. Oestrogen replacement therapy decreases abdominal fat [6] and increases bone density [7] in postmenopausal women. However, benefits of oestrogen replacement vs risk of progression of coronary artery atherosclerosis and diabetes are contentious [5, 8, 9]. Therefore, understanding mechanistic issues of links between visceral obesity and the metabolic syndrome would help devise interventions and derive site-specific as well as age-related benefits in menopausal women.

White adipose tissue (WAT) consists of several depots such as abdominal visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). VAT is distinguished from SAT by its anatomy and distinct secretion patterns of adipocytokines [10, 11]. Abdominal deep VAT, including mesenteric adipose tissue (MAT) in mice and omental fat in humans, exerts a more direct influence on liver metabolism. These secretions, collected by the portal vein, reach the liver at much higher concentrations than other visceral depots such as perigonadal and perirenal fat depots, whose veins drain into systemic circulation [11, 12]. Deep VAT is a major site of the chronic low-grade inflammation associated with obesity and also a sensitive insulin resistance predictor [13, 14]. Adipose tissue stromal vascular cells (SVC) in obese persons express increasing amounts of proinflammatory molecules such as *Tnf α* , *Il-6* and monocyte chemotactic protein (MCP)-1 [15]. Proinflammatory adipose tissue macrophages serve as a link between obesity and insulin resistance [16]. As an endocrine organ, adipose tissue and

the adipokines and cytokines derived from it play important roles in energy homeostasis, inflammation and insulin resistance [17]. In addition to known genes, 40% of genes expressed in adipose tissue are novel and 20–30% of these may encode secreted proteins requiring biochemical and physiological characterisation [18].

Follicle-stimulating hormone (FSH) receptor disruption produces chronic oestrogen deficiency and hyperandrogenaemia in female mice [19, 20]. In FSH receptor (*For* [also known as *Fshr*]) knockout mice, we previously drew attention to early obesity induced by hormonal imbalances: a low oestrogen:androgen ratio was associated with visceral obesity and onset of glucose intolerance, fatty liver and dyslipidaemia [21, 22]. This model allowed us to distinguish between deep VATs such as those surrounding mesenteric vessels and perigonadal adipose tissue (PAT) [22] and suggest that anatomical location causes distinct biological effects.

This led us to hypothesise that comparing abdominal adipose depots could reveal differences in the nature and/or quantity of these depots, and sex-dependent effectors. In the present work, we used *For*-knockout female mice to discover novel hormone-regulated, deep VAT-specific genes that are dysregulated and functionally involved in linking sex hormonal imbalances with obesity and insulin resistance. We show that a novel adipokine/proinflammatory cytokine, MEDA-7 (MEDA in Sanskrit to mean fat and standing for mesenteric oestrogen-dependent adipose gene 7 [*Meda-7*]), is hormonally regulated and implicated in contributing to chronic inflammation, cellular expansion and insulin resistance.

Methods

Animals and human tissue collection

Animals The animal care committee approved our study. *For*-knockout mice were established and housed as described [21, 22]. To test the effects of a high-fat diet (HFD), mice were fed a diet with 45% energy as fat from lard and soyabean oil [22].

Human participants Paired biopsies of SAT and omental fat were collected from obese patients during laparoscopic surgery. Investigations were approved by the hospital ethics committee and participants gave written informed consent.

Affymetrix gene microarray

RNA from MAT of 5-month-old wild-type or *For*-knockout mice was used. Analyses (Affymetrix 39 K GeneChip Mouse Genome 430 2.0 Array; Affymetrix, Santa Clara,

CA, USA) were done using equipment and protocols from the supplier. Data analysis was performed using GeneSpring software (Agilent Technologies, Mississauga, ON, Canada) with default normalisation [23].

RT-PCR, PCR and quantitative PCR

Expression of several genes in Figs 2 and 4 was determined by RT-PCR in relation to *GAPDH*. The sequences of specific PCR primers for RT-PCR and quantitative PCR are in the electronic supplementary material (ESM) Table. RT-PCR variables are described in ESM Method-1. Quantitative PCR was performed as described [22].

Isolation of full-length cDNA clones from mouse adipose tissue

Because expressed sequence tag (EST) of *Meda-7* is derived from the 3' end, an RNA ligase-mediated RACE system (FirstChoice; Ambion, Austin, TX, USA) was used to generate 5'-RACE PCR templates. PCR products were cloned into P driver vector (Qiagen, Mississauga, ON, Canada) and transformed into *Escherichia coli*. Isolated plasmid DNA was sequenced for assembly using Sequencer 4.7 software (Gene Codes Corporation, Ann Arbor, MI, USA).

Cloning and recombinant expression of mouse and human *MEDA-7* open reading frame

Both *MEDA-7* open reading frames (ORFs) were cloned into vector pIRES2 with green fluorescent protein (GFP) and V5 tag. GFP not fused to inserted protein allows monitoring of transfection efficiency. HEK-293 cells were transfected with *MEDA-7* ORF plasmid DNA using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). After 48 h, G418 (600 µg/ml) was added for selection. After 2 weeks, HEK-293 mouse or human *MEDA-7* ORF stable cell lines were established and expression confirmed by western blot. A stable clone of ovarian cancer UCI-107 cells expressing human *MEDA-7* ORF was also produced.

Western blotting and protein localisation

For western blot, proteins in cell lysate and supernatant fractions (50 µg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes [22]. *MEDA-7* antibody was preincubated with synthetic peptide (3 µg/ml) to determine specificity. β-Actin was used as a housekeeping gene. Protein localisation was determined by immunofluorescence (for details see ESM Method-2).

Isolation of SVC and adipocyte fraction, and macrophage sorting

The SVC and adipocyte fraction were obtained from PAT of 5-month-old wild-type female mice as described [15]. For cell sorting, pelleted erythrocyte-depleted SVCs from the wild-type PAT pool were resuspended in FACS buffer (PBS +0.2% [wt/vol.] fatty acid-poor BSA). Isolated cells were incubated with F4/80-APC and CD11c-FITC (eBioscience, San Diego, CA, USA) to sort macrophages into M1 (F4/80⁺ CD11c⁺) and M2 (F4/80⁺ CD11c⁻) phenotype [24], and processed for RNA.

Peritoneal macrophages

Thioglycolate-elicited peritoneal macrophages were collected from 3-month-old wild-type mice [25]. PBS-washed cells resuspended in RPMI 1640 supplemented with 10% FBS were plated (10⁶ cells/well). Non-adherent cells were removed after 2 h and medium was replaced. RNA was isolated from cells grown for 16 h in medium with or without lipopolysaccharide (LPS) 100 ng/ml.

Oestrogen replacement

Mice were treated with oestradiol-17β (1 µg/day for 10 days) [19] and MAT was processed for RNA.

Cell proliferation

HEK-293 cells were grown at 37°C under 5% CO₂ in DMEM+10% heat-inactivated serum. We analysed five variables: (1) cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen); (2) cell counting; (3) cell cycle: 1×10⁶ cells were fixed in 70% ethanol for 30 min at 4°C, then washed and resuspended in 1 ml PBS with 50 µg/ml propidium iodide and 20 µg/ml RNase A (Sigma-Aldrich, Oakville, ON, Canada), followed by FACS analysis (Becton Dickinson, Oxford, UK) using MODFIT LT software (Verity Software House, Topsham, ME, USA), resulting in 10,000 single events being recorded each time and percentage of cells in G1, G2 and S phases being calculated; (4) soft agar assay for colony formation by human *MEDA-7* ORF stably transfected HEK-293 cells and UCI-107 cells; and (5) western blot assessment of mitogen-activated protein kinase (MAPK) activity involved in cell proliferation.

Glucose uptake in adipocytes

Insulin-stimulated glucose uptake was measured [26] using differentiated 3T3-L1 cells. On day 7, recombinant

MEDA-7 (2 and 5 nmol/l) was added to cells for 20 h, followed by serum-free MEDA-7 or control media for an additional 4 h. Cells were treated for 20 min with or without 200 nmol/l insulin at 37°C and processed for glucose uptake, RNA and protein analysis. Membrane fractions were prepared according to published procedures [27] (see ESM Method-3 for details). Glucose uptake was initiated by 2-deoxy-D[1,2-³H]glucose addition (PerkinElmer, Woodbridge, ON, Canada) (37 kBq for 10 min at 37°C). Radioactivity in cell lysate was then determined. The method used to determine the concentration of recombinant MEDA-7 was based on a competitive ELISA assay using polyclonal antibodies against mouse MEDA-7 peptide (ESM Method-4).

Statistics

Results were analysed using SigmaStat3.1 (SYSTAT, Point Richmond, CA, USA). Values are presented as mean±SEM. The *t* test was used when comparing only one factor between two groups. Otherwise, we used ANOVA followed by Bonferroni's *t* test as specified. Statistical differences were considered significant at *p*<0.05.

Results

MAT transcriptome and discovery of novel gene *Meda-7*

To identify novel genes linking early visceral obesity and late onset of the metabolic syndrome in *For*-knockout females [21, 22], we compared MAT transcriptome in 5-month-old *For*-knockout and wild-type littermates. This identified 2186 dysregulated (≥twofold) transcripts. Using the DAVID Gene Functional Classification Tool (<http://david.abcc.ncifcrf.gov>), genes were clustered into functional groups (Fig. 1a). We focused exclusively on unknown genes, applying strict criteria to select genes for further study. First, using bio-informatics to deduce predicted hypothetical protein ORFs, we attributed significance if the gene was also predicted in humans. Second, we stipulated adipose tissue expression and disease association, as found in *For*-knockout. Third, we chose predicted proteins with features for functional analysis. Thus one EST identified by probe ID '1428947_at' was severely downregulated in *For*-knockout MAT to 8%. BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) matched with an uncharacterised transcript: *Mus musculus* RIKEN cDNA 2010001M09 gene. We named this *Meda-7*. Its alteration in *For*-knockout adipose tissue was confirmed by quantitative PCR. Thus *Meda-7* expression decreased to 8% at 5 months in *For*-knockout MAT (Fig. 1b) consistent with microarray data and tended to decrease in *For*-knockout PAT (Fig. 1c). However, by 9 months, when mutants become prediabetic

Fig. 1 Functional classification of dysregulated genes in *For*-knockout MAT. Transcriptome of adipose tissue from 5-month-old wild-type (WT) and *For*-knockout (*For*-KO) mice (*n*=3) was compared by Affymetrix microarray. **a** Dysregulated genes (≥twofold difference) were clustered into different functional groups, with 299 genes, including *Meda-7*, remaining uncharacterised. *Meda-7* expression by quantitative PCR at 5 months in wild-type and *For*-knockout mice in **(b)** MAT (***p*<0.01, *n*=8) and **(c)** PAT. *Meda-7* expression at 9 months in WT and *For*-KO (prediabetic state) in **(d)** MAT (***p*<0.01, *n*=8) and **(e)** PAT

[22], *Meda-7* was upregulated 8.6-fold in MAT (Fig. 1d) but not in PAT (Fig. 1e). Thus *Meda-7* was dysregulated in *For*-knockout females selectively in VAT in an age- and disease-related manner.

Cloning of mouse and human *MEDA-7* and *MEDA-7* protein characteristics

The mouse microarray probe matched to the 3' end of a predicted cDNA (NM_027222.2). We performed 5' RACE to extend the 5' region and assembled the full sequence. Sequence of adipose tissue mouse *Meda-7* cDNA showed four nucleotides to be different at positions 74, 130, 441 and 761. Three changes produce in-frame mismatches with predicted ORF: TTC (Phe) vs TTA (Leu), CCC (Pro) vs CAC (His) and AAT (Asn) vs AGT (Ser). These were confirmed in two other strains (C57/BL6 and Swiss Webster) prone to diet-induced obesity [28]. Mouse *Meda-7* gene mapping to chromosome 18B2 has four exons and three introns spanning 2,096 bp (ESM Fig. 1a).

Mouse *Meda-7* ORF codes for a protein of 188 amino acids.

We then cloned human adipose tissue *MEDA-7* ORF using primers at both ends. Sequencing confirmed 100% match to hypothetical protein LOC51237. Human *MEDA-7* gene mapping to chromosome 5q31 (ESM Fig. 1b) is located near a cluster of known cytokine genes such as *IL3*, *IL5*, *IL4*, *IL13*, *IL9* and *CXCL14*. Human *MEDA-7* cDNA codes for a 189 amino acid hypothetical protein MGC29506 (accession number NP_057543). Human *MEDA-7* shows high homology with other species (ESM Fig. 1c and d for accession number). Six cysteines are conserved in all species, a characteristic of many cytokines that form three disulfide bonds, including IL-17, MCP-3, chemokine (C-C motif) ligand (CCL)15, CCL21 and CCL23 [29–31]. Mouse and human *MEDA-7* contain an N-terminal signal peptide 1–22, which can yield a secreted protein (166 for mouse and 167 amino acids for human) (ESM Fig. 1e and f). Prediction tool CTKPred [32] suggested that mouse and human *MEDA-7* belong to the cytokine family.

The ‘Eukaryotic Linear Motif’ resource predicts several functional sites in human *MEDA-7* protein, including major TNF receptor-associated factor 2-binding motif [33, 34], signal transducer and activator of transcription (STAT) 5 Src-homology-2 domain binding [35, 36] and immunoreceptor tyrosine-based inhibitory motif [37], suggesting potential involvement in insulin signalling and inflammatory regulation.

Adipose tissue *MEDA-7* dynamics

In addition to high expression in MAT, *Meda-7* is also expressed in metabolic and reproductive organs of 3- to 5-month-old wild-type females, such as pancreas and kidney, followed by intermediate levels in PAT, uterus, SAT, heart, muscle, ovary and liver, and very low expression in brown adipose tissue (Fig. 2a). Analysis of *Meda-7* expression in PAT in mice (Fig. 2b) showed that *Meda-7* mRNA levels were significantly higher in SVCs than in adipocytes. Adiponectin (Fig. 2c) and *Tnfα* (Fig. 2d) expression was examined as markers of two fractions. Macrophages, as an important component of SVCs, contribute to chronic inflammation in obesity. *Meda-7* was expressed in mouse macrophage RAW 264.7 cell line, and *F4/80* and *Tnfα* were both detectable in these cells (Fig. 2e).

As paracrine macrophage–adipocyte cross-talk drives local and systemic insulin resistance [15, 17], we examined SVCs by FACS analysis. The macrophage (*F4/80*⁺ cells) component in *For*-knockout SVCs increased to 37% vs 15% in wild-type SVCs at 9 months, supporting macrophage involvement in the prediabetic state in *For*-knockout mice at this age. Macrophages are subgrouped into

proinflammatory M1 and non-inflammatory regulatory M2 phenotypes [38]. We sorted M1 phenotype (*F4/80*⁺, *CD11c*⁺ cells) and M2 phenotype (*F4/80*⁺, *CD11c*[−] cells) from SVCs of 9-month-old wild-type female PAT (Fig. 2f) and found *Meda-7* expression in both fractions, but consistently higher in the M1 fraction (Fig. 2g). The pattern for other M1-associated cytokines, e.g. *Il-6* and *Mcp-1*, was similar. Challenge with LPS, an endotoxin and Toll-like receptor-4 ligand, promotes secretion of proinflammatory macrophage cytokines [39]. LPS-stimulated peritoneal macrophages increased endogenous *Meda-7* mRNA (Fig. 2h) confirming its proinflammatory phenotype; control proinflammatory *Mcp-1* was also upregulated. Immunofluorescence using *MEDA-7* peptide antibody localised this protein in the cytoplasm of adipose tissue SVC fraction (Fig. 2i). A protein band of 22 kDa from SVC lysate by western blotting matched the predicted size (Fig. 2j); this was blocked by *MEDA-7* synthetic peptide antibody.

We next examined *Meda-7* in different adipose tissue depots as well as by sex and age. Expression was higher in WAT, including MAT, PAT and SAT (Fig. 2k), suggesting a primary function in energy-storing WAT as opposed to thermogenic brown adipose tissue. *Meda-7* in MAT differed between male and female mice (ESM Fig. 2a). In wild-type mice, *Meda-7* was 10 times higher in females than males, but there was no sex-related difference in *For*-knockout MAT. Examining *Meda-7* in MAT with ageing in females, we found a decrease in wild-type MAT. However, in *For*-knockout females, there was a clear trend for *Meda-7* expression to increase with ageing (ESM Fig. 2b). *Meda-7* expression was influenced by chronic HFD (Fig. 2l), increasing 125× in wild-type and 75× in *For*-knockout MAT on HFD compared with regular diet at 9 months.

MEDA-7 is a secreted protein

By western blotting, *MEDA-7* protein was found in cell lysate and culture medium of HEK-293 *Meda-7* ORF cells (molecular mass 22 kDa) (ESM Fig. 2c) confirming theoretical prediction of *MEDA-7* as a secreted protein. The protein band intensity in the culture medium indicated efficient secretion. Signal peptide (residues 1–22) deletion prevented secretion and the corresponding truncated protein was detectable only in cell lysate (not shown). Truncated *MEDA-7* is diffuse in the cytoplasm (ESM Fig. 2d).

MEDA-7 regulation by oestrogen

Since we initially discovered *MEDA-7* in MAT following dysregulation during sex hormonal imbalance, we verified oestrogen’s influence on gene expression. Treatment of 5-month-old *For*-knockout mice with oestradiol-17β had remarkable effects. Among the reproductive organs, the uterus

is most sensitive to oestrogen loss. This atrophic tissue in mutants recovered after oestradiol treatment [19]. At 5 months, *Meda-7* expression in *For*-knockout MAT, which had decreased to 8% as compared with wild-type ($p < 0.01$), was now reversed by oestrogen. Expression was five times higher in the *For*-knockout+oestradiol-17 β group than in the

For-knockout+placebo group (Fig. 3). Oestrogen had no effect on the expression of *Meda-7* in PAT (not shown), indicating that regulation at this age is restricted to selective abdominal fat depots. Oestrogen did not influence *Meda-7* expression in fat depots of *For*-knockout mutants at 11 months (not shown).

Fig. 2 Tissue expression of *Meda-7* in mouse. **a** *Meda-7* expression in different metabolic and selected reproductive organs was determined by quantitative PCR using specific primers and normalised to *Gapdh*. Wild-type (WT) female mice at 3–5 months of age ($n=3$) were used for this comparison. BAT, brown adipose tissue. **b** Compartmental expression of *Meda-7* in 9-month-old wild-type PAT in SVC vs adipocyte fraction (AF) by quantitative PCR, with (c) adiponectin and (d) *Tnfa* expression examined as markers of two fractions to confirm effective separation. **e** *Meda-7* expression in RAW 264.7 by PCR. *F4/80* and *Tnfa* mRNA were also detectable in the same samples. **f** Macrophages were sorted from SVC derived from PAT of 9-month-old wild-type mice. **g** Expression of *Meda-7* and two known proinflammation genes, *Mcp-1* and *Il-6*, in the fractions M1: *F4/80*⁺ and *CD11c*⁺ (R3) and M2: *F4/80*⁺ and *CD11c*[−] cells (R4). **h** Peritoneal macrophages were cultured overnight with and without LPS (100 ng/ml). *Meda-7* mRNA in macrophage was increased after LPS stimulation. Proinflammatory gene *Mcp-1* was also expressed and increased after LPS stimulation. **i** Immunofluorescence staining of MEDA-7 (white arrows) in PAT from 9-month-old wild-type mice. MEDA-7-producing cells in green were detected by MEDA-7 antibody and localised in SVC. Within the cell, the staining was localised in cytoplasm. Propidium iodide staining (red) indicates cell nucleus. Pre-immune normal rabbit serum was used as negative control. Scale bars 30 μ m. **j** Western blotting of MEDA-7 in adipose tissue. Cell lysate of SVC from PAT of 9-month-old wild-type mice were processed using MEDA-7 antibody (Ab) (1.5 μ g/ml; GenScript, Piscataway, NJ, USA). The 22 kDa band disappears after antibody incubation with peptide antigen. Anti-actin (1:6,000; Santa Cruz, Santa Cruz, CA, USA) was used as control for loading. **k** Different expression levels of *Meda-7* in different mouse fat depots. Four fat depots from 5-month-old wild-type female mice were compared by quantitative PCR. *Meda-7* expression was higher in white adipose tissue including PAT, MAT and SAT than in BAT; $n=4$. $**p<0.01$ by one-way ANOVA, $F=7.245$. **l** Effect of chronic HFD (white) on adipose tissue *Meda-7* expression in wild-type (WT) and *For*-knockout (*For*-KO) females by quantitative PCR analysis. At 9 months, HFD strongly influenced *Meda-7* expression (125-fold in wild-type, 75-fold in *For*-knockout MAT under HFD compared with regular diet [black]; $n=6$). $**p<0.01$ by two-way ANOVA, $F=24.07$. When comparing wild-type on regular diet with *For*-knockout on regular diet, there were significant increases in the latter; $n=6$; $p<0.05$

Characterisation of human adipose tissue MEDA-7

MEDA-7 was expressed in omental fat and SAT from obese patients (three women, one man), but levels in the former

Fig. 3 Oestrogen regulation of *Meda-7* expression in adipose tissue. Female mice (5-month-old) were treated with oestradiol-17 β (E₂) for 10 days. MAT was processed for RNA and expression determined by PCR; $n=5$, $*p<0.05$, $**p<0.01$

were three times higher (Fig. 4a). Previously an isoform of human MEDA-7 (called proapoptotic caspase-2- and caspase-9-binding protein [PACAP]) had been cloned and characterised as a proapoptotic adaptor protein with 123 residues [40]. However, when our adipose tissue studies began, neither human nor mouse MEDA-7 had been characterised. Due to a frame shift, the coding sequence is altered producing a transcript in which the first 59 N-terminal residues are identical and diverge thereafter [40, 41] (ESM Fig. 3). To differentiate these transcripts, we designed primers specific for MEDA-7 or PACAP (ESM Table). MEDA-7 was the predominant isoform in adipose tissue compared with PACAP. As a positive control for the primers, expression of PACAP and MEDA-7 was found in Jurkat cells (Fig. 4b).

Transfection of human MEDA-7 ORF in HEK-293 cells and western blotting (Fig. 4c) showed a single 22 kDa band in cell lysate and medium. Proteomic analysis confirmed structural composition of the secreted band in medium (data not shown).

MEDA-7 increases cell proliferation

In view of a previous report that the PACAP isoform induced apoptosis [40], we were intrigued by survival of HEK-293 cells used for transfection and protein confirmation for mouse and human MEDA-7. Stable cells showed higher growth rate than cells with vector DNA (Fig. 5a). Proliferation was confirmed by MTT assay (1.8 $\times$) (Fig. 5b). Cell cycle analysis showed a significant increase of diploid synthesis in HEK-293-MEDA-7 cells (Table 1). MEDA-7 had no effect on cell death. In addition, anchor-independent growth assay showed increasing colony formation after 2 weeks in HEK-293-MEDA-7 cells (1.4 $\times$) (Fig. 5c). Evaluating additional cell types, we found that human MEDA-7 in UCI-107, a human ovarian cancer cell line, significantly increased the number (1.5 $\times$) and size of colonies (Fig. 5d and e). Further probing MEDA-7 effects on proliferation, we evaluated MAPK as a potential intracellular signalling pathway. Robust activation of p44/42 MAPK was evident in cytosolic extracts of HEK-293-MEDA-7 stable clone (2.6 $\times$) (Fig. 5f and g). Therefore, MEDA-7 was able to mediate cell proliferation via p44/42 MAPK signalling.

Effect of MEDA-7 on insulin-dependent glucose uptake in adipocytes

We evaluated the effect of MEDA-7 on adipocyte insulin sensitivity by using differentiated 3T3-L1 adipocytes and verifying insulin-dependent glucose transport. Preadipocyte factor-1 (Fig. 6a) and adipocyte fatty acid-binding protein-2 (Fig. 6b) were tested as positive markers of undifferentiated

Fig. 4 Characterisation of *MEDA-7* in human adipose tissue. **a** *MEDA-7* expression assessed by quantitative PCR in SAT and omental fat depots (OMF) of obese patients; $n=4$; $*p<0.05$. **b** Relative expression of *MEDA-7* and its shorter form (*PACAP*, see text) in omental fat depot. *MEDA-7* is predominant in omental fat.

Jurkat cells used as positive control in PCR show higher *PACAP* expression. **c** Human *MEDA-7* ORF cloned in pIRES2V5-eGFP vector and transfected into HEK-293 cells shows efficient secretion of the processed protein. Western blot was performed using V5 antibody

and fully differentiated states on day 8. Insulin-dependent glucose uptake was reduced after incubation of cells with recombinant *MEDA-7* protein (2 and 5 nmol/l), while basal glucose uptake remained unchanged (Fig. 6c). This was associated with an increase of suppressor of cytokine signalling (SOCS)-3 (Fig. 6d) and *Mcp-1* (also known as *Ccl2*) mRNA (Fig. 6e) and reduction of *Glut4* (also known as *Slc2a4*) at mRNA (Fig. 6f) and protein levels (Fig. 6g). As a control for the fractioning process, β -actin was not detected from membrane fraction (Fig. 6g) and insulin receptor- β was barely detected in the cytosol fraction (Fig. 6h). GLUT4 protein was downregulated in total lysate (Fig. 6i) and membrane fraction (Fig. 6j) upon *MEDA-7* treatment after insulin stimulation. Level of the key downstream insulin signalling pathway protein, phospho AKT, was also downregulated (Fig. 6k). However, phospho insulin receptor- β and phospho IRS-1 were unaltered (data not shown). Therefore, *MEDA-7* contributed to the chronic inflammatory state of adipocytes and blunted insulin-dependent glucose uptake in adipocytes.

Discussion

Insulin resistance in obesity is closely related to the presence of inflammatory cells in adipose tissue, in particular a population of macrophages, which cause low-grade chronic inflammation and release various cytokines [14, 16]. Within the visceral area in female mice with hormonal imbalance, we previously showed that the inflammatory nature of MAT is different from that of the neighbouring perigonadal fat [22]. With the object of identifying the genes responsible for different effects, our study reports the cloning and characterisation of a novel gene, *Meda-7/MEDA-7*, in mice and humans. The changing dynamics of *Meda-7* expression in MAT in *For*-knockout mutants as they aged and approached a prediabetic state attracted our attention. In *For*-knockout mutants, *Meda-7*

was first downregulated at 5 months, when mice are in an early obese state with normal glucose tolerance. However, its upregulation at 9 months, in a prediabetic state, implied that *Meda-7* plays a role in advanced obesity and development of insulin resistance. As a secreted protein, *MEDA-7* is produced predominantly in adipose tissue SVC, which is composed of several cell types [11]. Its abundance in the macrophage M1 phenotype (Fig. 2g) supports an association with the chronic inflammatory state of adipose tissue and glucose intolerance in *For*-knockout mutants [22]. LPS-activated macrophages, which have been shown to increase proinflammatory cytokines [25] including *Tnf α* , *Il-1 β* and *Il-12*, also promote *Meda-7* expression. This also supported the proinflammatory phenotype of *MEDA-7*. *MEDA-7* secreted protein was able to decrease insulin-dependent glucose uptake in 3T3-L1 cells. This is associated with downregulation of GLUT4 and pAKT activity, and upregulation of *Mcp-1* and *Socs-3*, leading to blunting of insulin sensitivity in adipocytes [42, 43]. Therefore, *Meda-7* upregulation could contribute to the progression of insulin resistance in adipose tissue. In agreement with this, our previous study showed upregulation of other proinflammatory genes, e.g. *Tnf α* (also known as *Tnf*) and *Mcp-1*, in MAT of 9-month-old, but not in that of 5-month-old obese mutants [22]. Other studies have also confirmed that in obesity, overproduction of macrophage-derived cytokines causes insulin resistance by downregulating *Glut4* [44].

MAT in rodents resembles human omental fat. Both are major abdominal fat depots showing a causal relationship between visceral obesity and insulin resistance. MAT is a better insulin sensitivity marker than other abdominal fat depots [13]. *Meda-7* dysregulation in mutant MAT is greater than in PAT, which is also consistent with our previous findings of prominent alterations of anti/pro insulin resistance adipokines, including adiponectin, *Tnf α* and *Mcp-1* in MAT [22]. *MEDA-7* abundance in obese humans also varies according to adipose tissue location, being higher in omental fat than SAT (Fig. 4a). Thus, based

Fig. 5 *MEDA-7* increases cell proliferation. **a** Cells (3×10^5) were plated in each dish and cell number counted at indicated times. Trypan blue staining was used to indicate dead cells. $n=4$, $*p<0.05$. Triangles, HEK-293 vector; squares, HEK-293 *MEDA-7*. **b** Cells were plated in 96-well plates (10^4 /well) for 48 h and processed by MTT assay; absorbance was observed at 570 nm. $n=4$, $*p<0.05$. White, HEK-293 vector; black, HEK-293 *MEDA-7*. **c** Colony formation in HEK-293 human *MEDA-7* cells (black bars) vs HEK-293 vector (white bars). $n=3$, $*p<0.05$. **d** Images of colony formation in UCI-107 ovarian cancer cells transfected with human *MEDA-7*

ORF, with **(e)** quantification. Cells were seeded in triplicate at a density of 1×10^5 per 3.5 cm dishes in 0.3% (wt/vol.) soft agar medium. After 2 weeks growth, the cells were stained with crystal violet (0.005%, wt/vol.). The number of colonies were counted and expressed per field of view; $n=3$, $*p<0.05$. White, UCI-107 vector; black, UCI-107 *MEDA-7*. **f** p42/44 MAPK activity in HEK-293 human *MEDA-7*. Representative western blots probed with phospho-p44/42 MAPK antibody and with 44/42 MAPK antibody are shown. Activity of p44/42 MAPK was normalised to total 44/42 MAPK. **g** Quantification of blots; $n=3$, $*p<0.05$

Table 1 Cell cycle analysis in HEK293-*MEDA-7* cells

Variable	G1	S	G2
HEK293-vector	57.5±2.0	30.13±4.0	12.37±2.4
HEK293- <i>MEDA-7</i>	54.1±2.6	36.76±4.4*	9.2±2.0

Values are mean±SEM, $n=3$

* $p<0.05$

on findings in mice and obese humans, we attribute significance to the high abundance of *MEDA-7* in deep VAT.

Reversal of *Meda-7* downregulation in *For*-knockout mouse adipose tissue by oestrogen at an early age but not later confirms its role as an important age-dependent regulator (Fig. 3). As transcription factor prediction by multiTF program (<http://dcode.org>) indicated that mouse and human *MEDA-7* promoter regions contain an oestrogen receptor binding site, we suggest that part of the regulation occurs at the transcriptional level. Failure of oestrogen to

Fig. 6 Effect of secreted MEDA-7 (recombinant) on insulin action in murine adipocytes in culture. **a** By quantitative PCR, *Pref-1* (also known as *Dlk1*) and **(b)** *Ap2* (also known as *Fabp4*) mRNAs were measured in 3T3-L1-D8, confirming differentiation process. **c** Insulin-stimulated glucose uptake was determined in differentiated 3T3-L1 cells pretreated with culture medium containing secreted human MEDA-7 (2 nmol/l and 5 nmol/l) vs media from HEK-293-vector cells, which served as control protein. Basal glucose uptake was unchanged; $n=4$. **d** In 3T3-L1 adipocytes treated by recombinant MEDA-7, *Socs3*, **(e)** *Mcp-1* and **(f)** *Glut4* mRNA was measured by

quantitative PCR; $n=6$. **g** GLUT4 protein level was measured in plasma membrane and total cell lysate. Representative western blots are shown. β -Actin was not detected from membrane fraction. **h** Insulin receptor (IR)- β was barely detected in the cytosol fraction, confirming the fractionation process. Band density was quantified after probing with GLUT4 antibody in **(i)** total lysate and **(j)** membrane fraction; $n=4$. **k** Phospho AKT (pAKT) activity was measured in total cell lysate. Representative western blots probed with pAKT and AKT antibodies are shown. pAKT was normalised to total AKT and quantified; $n=4$. * $p<0.05$ and ** $p<0.01$

modulate *Meda-7* at later ages could be attributable to a decrease of oestrogen receptor- α in MAT in *For*-knockout

mice after prolonged oestrogen deficiency [22]. Our results are supported by other studies in animal models or human

participants, which indicate that timing is critical to deriving benefits from hormone replacement. Specifically, beneficial effects of oestrogen replacement on risk of coronary events and progression of atherosclerosis are evident when therapy is initiated early in the postmenopausal period, but these are lost in participants who have pre-existing coronary artery atherosclerosis and are several years beyond the onset of menopause [45, 46].

There appears to be a single transcript of the *Meda-7* gene, as our cloning showed a single band that was identified as *Meda-7* cDNA in mice. However, human *MEDA-7* has one additional isoform that has been previously cloned and characterised as *PACAP* [40]. This PACAP protein, binding to caspase-2 and caspase-9, induced apoptosis in HEK-293 cells and rat-1 fibroblasts. A frame shift caused by alternative splicing produced this transcript.

Its low expression in obese human adipose tissue (Fig. 4b) compared with the levels of *MEDA-7* mRNA and the corresponding protein suggests that PACAP protein is unlikely to be of biological significance in this setting. For several reasons, we point out that using the *PACAP* acronym for *MEDA-7* variant (EST AA769445) is erroneous and misleading, as another, better characterised molecule, the pituitary adenylate cyclase activating peptide (the original *PACAP* and redesignated *ADCYAP1*), had been identified much earlier and extensively investigated for its neuroendocrine function and regulation, including a role in energy metabolism [47]. As indicated by its effects on HEK-293 cells, neither mouse nor human *MEDA-7* protein is deleterious for cell survival or growth. On the contrary, both have significant cell-stimulating activity via activation of MAPK (Fig. 5g). This conclusion is also supported by

Fig. 7 Mechanisms linking visceral obesity and insulin resistance in *For*-knockout (*For*-KO) female mice. Chronic hormonal imbalance consisting of combined oestrogen deficiency and androgen excess causes visceral obesity and macrophage activation. As the inflammatory state intensifies, *Meda-7* among other factors is upregulated by 9 months to cause cellular expansion via robust activation of pMAPK44/42 and blunting of insulin response in adipocytes by decrease of GLUT4, increase of *Mcp-1* and *Socs-3* abundance, and reduction of pAKT activity. *Meda-7* downregulation in MAT of *For*

knockouts can be reversed by oestrogen at 5 months, but the effect is lost at 9 months, by which time oestrogen receptor (ER)- α abundance in adipose tissue is also diminished. As shown previously [21, 22], *For*-knockout females acquire insulin resistance and the metabolic syndrome characteristics by 12 months. HFD causes pronounced upregulation of *Meda-7* expression in the adipose tissue, exacerbating the inflammatory state. CCR2, chemokine (C-C motif) receptor 2. E₂, oestradiol-17 β

enhanced anchorage-independent growth in HEK-293 and UCI-107 ovarian cancer cells.

The gene corresponding to MEDA-7 is also known as MGC 29506, which is apparently downregulated in intestinal-type gastric cancer [41]. The gene's functional significance in this context is unknown. Our study is the first to clone *Meda-7* ORF from mouse and human adipose tissue. As our findings indicate that *Meda-7* is also expressed in other metabolically active organs such as the heart, muscle, kidney and pancreas (Fig. 2a) in mice, the novel protein may have additional roles in different targets. Therefore cellular context-dependent functions of MEDA-7 are possible. Thus when we had completed our MEDA-7 studies in adipose tissue and obesity and were in the process of preparing this manuscript, two papers reporting a similar transcript and identifying an endoplasmic resident protein named pERp1 in the immune system came to our attention. This entity has been attributed a chaperone function during immunoglobulin assembly [48, 49].

In conclusion, our studies identified a novel secreted protein called MEDA-7, which is produced in mouse and human adipose tissue and is regulated by oestrogen in a fat depot- and age-dependent manner. MEDA-7, as a new proinflammatory cytokine, provides an additional link between obesity and onset of insulin resistance. We visualise a scenario (Fig. 7) in which MEDA-7 upregulation occurs at later ages in *For*-knockout female mice with hormonal imbalances associated with chronic inflammatory states, and progression to local and systemic insulin resistance in mutants. MEDA-7 predominantly expressed in SVC and localised in proinflammatory M1 macrophages has a direct effect on glucose uptake triggered by insulin in adipocytes. MEDA-7 also promotes cell growth in other cell types such as ovarian cancer. Thus MEDA-7 is a novel hormonally regulated adipokine/cytokine implicated in causing chronic inflammation, controlling cellular expansion and contributing to development of insulin resistance.

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HZ designed the study, performed experiments, researched data and wrote the manuscript. XC researched and analysed data. MRS designed the study, researched data, contributed to discussions and reviewed and edited the manuscript. All authors have approved this final version.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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