Novel Genes of Visceral Adiposity: Identification of Mouse and Human Mesenteric Estrogen-Dependent Adipose (MEDA)-4 Gene and Its Adipogenic Function

H. Zhang, X. Chen, and M. R. Sairam

Molecular Endocrinology Laboratory (H.Z., X.C., M.R.S.), Institut de Recherches Cliniques de Montréal, Montréal, Quebec, H2W 1R7 Canada; Département de Médecine (M.R.S.), Université de Montréal, Montréal, Québec, H3T 1J4 Canada; Department of Medicine (M.R.S.), Division of Experimental Medicine & Department of Physiology (M.R.S.), McGill University, Montréal, Québec, H3G 1Y6 Canada

Visceral adiposity represents a high risk factor for type 2 diabetes, metabolic syndrome, and cardiovascular disease as well as various cancers. While studying sex hormone imbalance-induced early obesity and late onset of insulin resistance in FSH receptor knock out female mice, we identified a novel mesenteric estrogen-dependent adipose gene (MEDA-4) selectively up-regulated in a depot-specific manner in mesenteric adipose tissue. Meda-4 cloned from both mouse and human adipose tissue codes for a 34-kDa cytosolic protein with 91% homology. Mouse Meda-4 mRNA is expressed highest in visceral adipose tissue and localizes predominantly in the adipocyte fraction. Human MEDA-4 is also more abundant in omental fat than sc depot in obese patients. In 3T3-L1 cells endogenous Meda-4 expression increases early during differentiation, and its overexpression promotes differentiation of preadipocytes into adipocytes and enhances glucose uptake. Conversely, short hairpin RNA-mediated knockdown of Meda-4 reduces both adipogenic and glucose uptake potential. In promoting adipogenesis, Meda-4 up-regulates transcription factor peroxisome proliferator-activated receptor-γ2. Meda-4 promotes lipid accumulation in adipocytes, regulating adipocyte fatty acid-binding protein 2, CD36, lipoprotein lipase, hormone-sensitive lipase, acyl-Coenzyme A oxidase-1, perilipin-1, and fatty acid synthase expression. 17β-Estradiol reduced Meda-4 expression in mesenteric adipose tissue of ovariectomized mice and in 3T3-L1 adipocytes. Thus our study identifies Meda-4 as a novel adipogenic gene, capable of promoting differentiation of preadipocytes into adipocytes, increasing lipid content and glucose uptake in adipocytes. Therefore it might play an important role in adipose tissue expansion in normal and aberrant hormonal conditions and pathophysiological states. (Endocrinology 153: 2665–2676, 2012)

In obesity, excess visceral adipose tissue accumulation in both sexes is associated with adipocyte dysfunction, manifested as abnormalities in adipokines and induction of a chronic proinflammatory state that could lead to systemic insulin resistance and metabolic syndrome (1). This ultimately increases the risk of type 2 diabetes and cardiovascular disease, which are also among the leading causes of death in postmenopausal women (2, 3). The prevalence of these conditions in postmenopausal women suggests that increases in visceral (central abdominal) obesity (4, 5) related to loss of ovarian function may contribute to the higher incidence of postmenopausal cardiovascular disease. In addition, epidemiological studies also demonstrate a close link between obesity and rising incidence of various cancers (6). Thus unraveling the complexity of molecular events underlying the determination and differentiation status of adipose tissue depots in states related to altered hormonal status is of basic and potential clinical importance.

Abbreviations: AF, Adipocyte fraction; aP2, adipocyte fatty acid-binding protein 2; C/EBP, CCAAT/enhancer-binding protein; E2, 17β-estradiol; FBS, fetal bovine serum; FORKO, FSH receptor knockout mutant; GFP, green fluorescent protein; HEK, human embryonic kidney; MAT, mesenteric adipose tissue; MEDA, mesenteric estrogen-dependent adipose gene; OMA, omental adipose tissue; ORF, open reading frame; O VX, ovaricetomized; PAT, peritumoral adipose tissue; PPAR, peroxisome proliferator-activated receptor; Pref-1, preadipocyte factor-1; Q-PCR, quantitative PCR; RACE, rapid amplification of cDNA ends; SAT, sc adipose tissue; shRNA, short hairpin RNA; WT, wild type.

Endocrinology, June 2012, 153(6):2665–2676 endo.endojournals.org

2665
Adipogenesis is the coordinated process of cell differentiation by which preadipocytes become lipid-laden adipocytes and secrete several bioregulators and create a new microenvironment. Currently, peroxisome proliferator-activated receptor (PPAR) γ (7) and CCAAT/enhancer-binding proteins (C/EBP) (8) have emerged as master regulators of adipocyte development. Many other important players such as wingless-type/b-catenin pathway (9), early-B-cell factor 1 (10), and sterol-regulatory element-binding transcription factor-1 (8) also integrate and regulate adipocyte differentiation. These findings suggest that fat cell development is a dynamic process requiring integration of multiple regulatory factors to determine the ultimate differentiation and functional state of the mature adipocyte.

Adipose tissue is a major site of peripheral aromatization of androgens to estrogens after menopause (11). Adipose tissues also express receptors for sex steroid hormones in an age-dependent manner. Alteration in sex steroids in turn impact on adipose tissue storage and functions both in humans and experimental animals (12). Several animal studies, including FSH receptor knockout mutants (FORKO mice) (13, 14), estrogen receptor-α knockout mutants (15), and the aromatase knockout mice (16) demonstrate that deficiency in estrogen and/or estrogen receptor produce weight gain, increased visceral adiposity, and impaired glucose/insulin tolerance. However, the mechanisms and genes responsible for altering adipose (depot) tissue behavior in these instances are not completely known. After genetic ablation of the FSH receptor in female mice, circulating estrogen becomes very low as early as 24 d, whereas its precursor testosterone remains significantly elevated at all ages studied (17). Thus simulating estrogen loss coupled with hyperandrogenemia in a gene dose-dependent manner induced obesity in females (14), resulting in clusters of phenotypes that mimic many complications found in postmenopausal women. Visceral obesity developing as early as 3 months progressively escalates at 9-months in FORKO females (13, 14, 18), when metabolic disorder phenotypes, including glucose intolerance, fatty liver, and dyslipidemia emerge. These metabolic changes are also associated with aberrations in adiponectin signaling and inflammatory gene expressions in adipose tissue in a fat depot-specific manner (18). Several genes are more dramatically dysregulated selectively in the mesenteric adipose tissue (MAT), a more central abdominal fat depot than the peritoneal adipose tissue (PAT) that is also a superficial visceral fat depot. Such a discriminatory expression profile is in accordance with the idea that central abdominal fat plays a determinant role in metabolism because its secretions are drained directly into the portal system, and lipolysis in central abdominal fat is less suppressible by insulin (19, 20). These interesting disease- and chronology-related features prompted us to explore and identify novel hormone-regulated deep visceral fat depot-specific genes that are dysregulated in FORKO mutants and that might contribute to sex hormonal imbalance-induced obesity and manifestation of insulin resistance with aging. Characterizing these novel genes in human obesity could also contribute to a greater understanding of their implications for causing or altering course of disease.

Microarray screening of genes altered in MAT of adult obese FORKO females compared with normal wild-type (WT) animals of the same age led us to focus on unknown genes. Using this tool, we recently identified a novel mesenteric estrogen dependent adipose gene (Meda)-7, as a proinflammatory cytokine, which is dysregulated in MAT of obese FORKO females, and demonstrated its role in the onset of insulin resistance (21). We herein report the cloning and characterization of another novel gene called Meda-4, from both mouse and human visceral adipose tissue and provide evidence for its involvement in adipocyte differentiation and adipogenesis. Its dysregulation early in FORKO obese female mice in a depot-specific manner (and regulation by estrogen), as well higher expression in omental fat compared with sc depot in severely obese patients suggests an important contribution to visceral adiposity.

Materials and Methods

Animals and human tissue collections

Animals. Female mice were used for all experiments, and males were included in gender comparisons where pertinent. Animal study was approved by the animal care committee of the Institut de Recherches Cliniques de Montréal. FORKO mice were established and housed as previously described (13, 18). All comparisons were performed using age-matched littersmates.

Human subjects. To compare human MEDA-4 expression in fat depots, we used paired biopsies of sc adipose tissue (SAT) and omental adipose tissue (OMA) that could be obtained from obese patients during laparoscopic bariatric surgery. Investigations were approved by the Hotel Dieu Hospital Montreal Ethics Committee with written informed consent from the patients.

Affymetrix gene microarray

For microarray analysis, RNA from dissected MAT collected from 5-month-old WT or FORKO mice (n = 3) were used. Affymetrix 39 K GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA) analyze more than 39,000 transcripts of mouse genes. Gene chip expression analyses were performed using Affymetrix equipment and protocols. Data analysis was performed using GeneSpring software (Agilent Technologies,
Quantitative PCR (Q-PCR)

Q-PCR was performed using gene-specific primers (Supplementary Table 1 published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org) as previously described (18).

Gene cloning

To obtain the full-length 5′- and 3′-ends of cDNA based on sequence of expressed sequence tags, FirstChoice RNA ligase-mediated-RACE (rapid amplification of cDNA ends) system (Ambion, Inc., Austin, TX) was used to generate 5′-RACE PCR templates and 3′-RACE PCR template as per instructions. PCR products were cloned into pDriver vector and transformed into *Escherichia coli*. Isolated plasmid DNA was sequenced for assembly using sequencer 4.7 software.

Cloning and recombinant expression of mouse *Meda-4* open reading frame (ORF) or human *MEDA-4 ORF*

A cDNA fragment containing the ORF of mouse *Meda-4* was cloned into vector pIRE2 (CLONTECH Laboratories, Inc., Palo Alto, CA) with green fluorescent protein (GFP) and V5 tag. In this expression system GFP tag is not fused to the inserted protein but allows monitoring cell transfection efficiency. Cells [human embryonic kidney (HEK)293 and 3T3-L1 preadipocytes] were transfected with mouse *Meda-4* ORF plasmid DNA or human *MEDA-4* ORF plasmid DNA using Lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada). After 48 h, G418 (600 μg/ml for HEK293 and 1000 μg/ml for 3T3-L1 cells) was added for selection. After 2 wk, stable cell lines were established and expression confirmed by Western blot using V5 antibody.

Short hairpin RNA for *Meda-4*

The oligonucleotide sequences used to create pSUPER-Retro-short hairpin RNA (shRNA) *Meda-4* or scrambled control (23) (Supplemental Table 1) were annealed and ligated into pSuper.Retro.Neo.Gfp (OligoEngine, Seattle, WA) as per suggested guidelines. Successfully isolated clones were verified by sequencing. 3T3-L1 cells were transfected with constructs using Lipofectamine 2000. After 48 h, G418 (1000 μg/ml) was added for selection. Clones were subsequently identified 2 wk later and stable cell lines were established. Expression of *Meda-4* in these selected 3T3-L1 subclones were evaluated by Q-PCR.

Western blot extracts and immunohistochemistry

Whole-cell extracts were prepared, and Western blot analysis with mouse Anti V5 (1:5000, Invitrogen) performed as reported elsewhere (18). For immunohistochemistry, the cells grown on slides were fixed in 3% fresh paraformaldehyde and permeabilized in 0.2% TritonX-100 and treated with 3% H2O2. Mouse Anti-V5 (1:500) or normal rabbit serum (negative control) was added and incubated overnight at 4 C, followed by another incubation for 1 h with horseradish peroxidase-conjugated antimouse antibody (1:800, Sigma-Aldrich, Oakville, Ontario, Canada). Signals were amplified with avidin-biotinylated horseradish peroxidase developed with diaminobenzidine.

Adipose tissue fractionation

To facilitate rapid processing and analysis, the stromal vascular fraction and adipocyte fractions (AF) were obtained from the more abundant PAT of 5-month-old WT female mice as previously described (24). Total RNA was extracted from the two fractions.

3T3-L1 cell differentiation and Oil-red-O staining

3T3-L1 cells representing preadipocytes were grown in 10% fetal bovine serum (FBS)-DMEM at 37 C in 5% CO2. For differentiation, preadipocytes were grown to 100% confluence in 10% FBS-DMEM. Two days after reaching confluence (d 0), the cells were stimulated with 5 μg/ml human insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine. After 3 d (d 3), medium was changed, and cells were treated with 5 μg/ml human insulin. Then, on d 6, medium was changed to 10% FBS-DMEM and full differentiation is achieved by day 7–8. For Ppar γ ligand treatment, pioglitazone 5 μM (Eli Lilly Canada, Inc., Toronto, Ontario, Canada) was used to treat cells from d 0 for 3 d. 3T3-L1-*Meda-4*-expressing cells and 3T3-L1-vector cells were differentiated under identical conditions and stained for lipid content using Oil Red O. Quantification of the dye extracted by 2-propanol was carried out as described elsewhere (25) by measuring optical density at 510 nm.

Ovariectomy and 17β-estradiol (E2) replacement

WT female mice (5-month old) were divided into three groups. Group 1: sham operated. Group 2: ovariectomized (OVX). Group 3: OVX + 10-d E2 treatment (OVX + E2). Bilateral ovariectomy was performed as described previously (26). From d 14–23, E2 (1 μg/d dissolved in olive oil) was injected sc for group 3; vehicle was injected for groups 1 and 2. On d 25, uterine and body weights were recorded, and MAT was processed for RNA.

Acute 17β-estradiol treatment of 3T3-L1 cells

3T3-L1 differentiated adipocytes (d 8) previously cultured in medium was transferred to serum-free conditions. E2 (0, 20 nM, or 2 μM) was added 8 h later to the serum-free medium. After 16 h, cells were processed for RNA isolation and *Meda-4* expression was analyzed by Q-PCR. In the time course study, 3T3-L1 adipocytes were treated with 200 nM E2 for 8 h, 16 h, and 24 h after 8 h of serum starvation.

Glucose uptake in 3T3-L1 cells

Insulin-stimulated glucose uptake was measured as previously described (27) using fully differentiated 3T3-L1-*MEDA-4* stable cells and 3T3-L1-Vector cells on d 8. Briefly, the cells were incubated with serum-free DMEM medium for 4 h followed by treatment with or without 200 nM insulin for 20 min at 37 C. Uptake was initiated by addition 2-deoxy-d-[1,2-3H] glucose to a final concentration of 1 μCi for 10 min at 37 C. Cells were washed and then lysed for counting the incorporated radioactivity.
Statistical analyses

Statistical analyses were performed using SigmaStat3.1 (SYSTAT, Point Richmond, CA). Values are presented as means ± SEM. The significance of the results was determined by using one-way ANOVA, two-way ANOVA, or three-way ANOVA as specified in figure legends followed by Bonferroni t test for multiple comparisons. For the remainder, the t test was used. Statistical differences were considered significant at P < 0.05.

Results

Transcriptome analysis of VAT and discovery novel gene Meda-4

We sought to identify hormone imbalance-induced dysregulation of deep visceral fat-specific genes that could be functionally implicated in development of obesity and insulin resistance in our mutants. We reasoned that such genes could be dysregulated (up- or down-regulated) in vivo, whereas visceral obesity became prominent in FORKO mice at 5 months of age. By Affymetrix Mouse Genome 430 2.0 Array, we performed transcriptome analysis in deep visceral adipose tissue-MAT in 5-month-old FORKO female and WT littermates. The significance analysis of microarrays procedure identified 974 known transcripts and 181 unknown transcripts that are dysregulated more than 3-fold, P < 0.05, as compared with WT littermates. Although we used this conservative and higher cutoff to limit the number of altered genes for further consideration, it does not preclude the importance of gene changes that were less affected in our model. By using DAVID Gene Functional Classification Tool (http://david.abcc.ncifcrf.gov), the known gene alterations were subdivided into different functional groups, an example of which is shown in supplemental Table 2A. We focused on genes listed as expressed sequence tag (EST) or having unknown function for further investigation. Strict criteria were applied to select genes for characterization. First, using bioinformatics and data banks to deduce predicted ORF for hypothetical proteins, we attributed significance to the ORF, only if the gene’s existence was also predicted in human genome suggesting conserved function. Second, we stipulated adipose expression and disease association, as was the case in FORKO adipose tissue. Third, we chose predicted proteins showing potential conserved functional domains that could help design functional studies. Based on these criteria, we found an expressed sequence tag with Affymetrix probe ID 1452244_at was selectively up-regulated 4.5-fold (P = 0.009), in FORKO MAT, as compared with the corresponding fat depot in WT mice. To obtain initial sequence information, BLASTN search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) matched a predicted uncharacterized transcript: Mus musculus RIKEN cDNA 6330406I15 gene. We named the gene as Meda-4. Meda-4 mRNA expression was then verified by RT-PCR and Q-PCR analysis with a different batch of adipose tissue samples. Our results confirmed that Meda-4 expression increased 5-fold (P < 0.05) in 5-month-old FORKO MAT (Fig. 1A). However, no significant change was evident in PAT from the same animals (Fig. 1B), indicating selective visceral depot-specific gene regulation. Meda-4 expression was also detected by Q-PCR among several mouse tissues (WT at 5-months). In both sexes (Fig. 1, C and D), Meda-4 mRNA was highly expressed in WAT (white adipose tissue) compared with other organs. In mice significant Meda-4 expression was also noted in the heart, brain, and pancreas with a clear trend toward sexual dichotomy in these organs.

To investigate MEDA-4 expression in human adipose tissue depots, we performed an initial screening of a limited number of fat depot samples from obese humans. Using Q-PCR we quantified MEDA-4 expression in paired OMA and SAT of these obese patients undergoing bariatric surgery [three women (ages 22, 40, 56) and one male (age 55), body mass index: 37–51]. In all cases MEDA-4 expression was higher in OMA than SAT (Fig. 1E). Based on these results, we concluded that Meda-4 is highly expressed in the visceral fat depot of both mice and humans. To verify whether the Meda-4 could be effectively translated into protein, both mouse and human MEDA-4 ORF were cloned into pIRES2–V5-GFP and transfected into HEK293 cells. Using V5 tag antibody, mouse MEDA-4 was detected in HEK293-mouse Meda-4 cell lysate (34 kDa) by Western blot, and immunohistochemical staining indicated cytoplasmic localization of the protein (Fig. 1F). Similar results were observed in HEK293-human MEDA-4 transfected cells (Fig. 1G). No bands were detected in the culture media (data not shown). Prosite Motif search (prosite.expasy.org) predicted six different protein domains of potential posttranslational modifications as shown in Supplemental Table 3.

Characterization of Meda-4 in mouse adipose tissue

Q-PCR analysis in WT 5-month females (Fig. 2A) and males (Fig. 2B) indicated that Meda-4 mRNA was higher in WAT (including PAT, MAT, and SAT), than brown adipose tissue, suggesting a primary function for Meda-4 in energy-storing adipocytes as opposed to thermogenic ones (28). As shown in Fig. 2C, significant differences were noted between genders in mice. Meda-4 increased in FORKO mice in a gender- and fat depot-specific manner (only in female MAT) with no dramatic differences in males. Meda-4 was higher in mature adipocyte fraction than in preadipocytes containing stromal/vascular frac-
human MEDA-4 localization of mouse MEDA-4 and human MEDA-4. *, immunostaining with V5 tag antibody indicated protein expression (34 kDa) and intracellular expression in SAT and OMA depots of obese patients were determined by Q-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 5 months of age were determined by Q-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 

FIG. 1. Meda-4 regulation and expression. Meda-4 mRNA expression in 5-month-old female FORKO obese MAT (A) and PAT (B) as compared with WT littermates (n = 7) by Q-PCR. Tissue expression of Meda-4 in different organs in female (C) and male (D) SV129 WT mice (n = 3) at 5 months of age were determined by Q-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Epididymal fat in males was studied. E, Meda-4 expression in SAT and OMA depots of obese patients were determined by Q-PCR and normalized to GAPDH (n = 4; three women and one man). Mouse Meda-4 ORF (panel F) and human Meda-4 ORF (panel G) transfected in HEK293 cells and detected by Western blot and immunostaining with V5 tag antibody indicated protein expression (34 kDa) and intracellular localization of mouse Meda-4 and human Meda-4. *, P < 0.05; **, P < 0.01.

Effect of Meda-4 on adipocyte differentiation and lipid storage

Because Meda-4 is predominantly found in the WAT with higher expression in mature adipocyte fraction and increased in FORKO obese MAT, we hypothesized that the mature adipocyte is a source as well as a target of Meda-4 action. We tested this hypothesis using the 3T3-L1 adipocyte model in cell culture (29). Meda-4 was expressed in undifferentiated 3T3-L1 cells and increased during differentiation around d 5 (middle period of differentiation) and further increased at d 8 (fully differentiated stage) (Fig. 3A), suggesting its involvement in adipogenesis. In these studies the expression of preadipocyte factor-1 (Pref-1) (30) (Fig. 3B) and adipocyte fatty acid-binding protein-2 (aP2) (Fig. 3C) (31) were also verified as positive markers for the undifferentiated and fully differentiated states.

To further explore Meda-4 effect on adipocyte phenotypes, 3T3-L1 cells that stably overexpress mouse Meda-4 ORF were generated. By Q-PCR, Meda-4 was expressed 4.2 fold higher in 3T3-L1-Meda-4 cells as compared with

Cloning of Meda-4 gene

Meda-4 full-length cDNA was cloned from mouse adipose tissue by 5’-RACE and 3’-RACE with gene-specific primers derived from Affymetrix probe ID 1452244_at (Supplemental Fig. 1A). The full cDNA sequence assembled from at least four clones matches 100% with the predicted sequence with accession number NM_027519. Meda-4 gene is predicted to have five exons and four introns spanning 19.9 kb. The predicted ORF located at position 173-1084 from 5’-end of cDNA with start codon ATG and stop codon TGA. Thus Meda-4 gene encodes a protein of 303 amino acids. Meda-4 gene maps to murine chromosome 5G3 whereas the human MEDA-4 maps to chromosome 13q12 (Supplemental Fig. 1B). Human MEDA-4 ORF was also cloned from human adipose tissue by PCR using primers including the predicted start and stop codons (Supplemental Fig. 1C). Sequencing of four different clones confirmed that comparing our sequence with predicted MEDA-4 in the human genome, there are three single nucleotide mismatches: at position 68 (from 5’-end of human MEDA-4 ORF): A replacing G produces Histidine (cAc) instead of Arginine (cGc); positions 174 and 624: T replacing C are silent variants. The amino acid sequence of human MEDA-4 deduced from cloning shows a sequence identity of 99%, 91%, 91%, and 90% with sequences predicted for chimpanzee, rat, mouse (our cloning), and cow orthologs, respectively (Supplemental Fig. 2, A and B).
control (Fig. 3D). This is quite comparable with the level of change occurring in FORKO MAT (in vivo) assuring this experimental manipulation as a useful tool with which to study the function of elevated Meda-4 in vitro. After differentiation (d 8) and visualization by Oil-red O staining, these 3T3-L1-Meda-4 cells accumulated more lipid droplets, as compared with control cells with vector (Fig. 3E).

Because Meda-4 overexpression led to an increase in lipid accumulation in adipocytes, we also examined the effect on glucose uptake by testing adipocytes after differentiation (d 8); overexpression of Meda-4 increased both basal and insulin-stimulated glucose uptake (Fig. 3F). Probing of underlying molecular events revealed that Meda-4 overexpression caused up-regulation of several key players of adipogenesis: Ppar-γ, Ppar-γ2, and C/EBP-α. Among these, Ppar-γ2 is a well-established adipocyte-specific isoform of Ppar-γ. In addition, the upstream regulator C/EBP-α was also increased. Correspondingly, Pref-1, a negative regulator of adipogenesis (32), was down-regulated. These patterns suggested that Meda-4 promotes adipogenesis by regulating key transcription factors. In addition, Meda-4 also promoted lipid storage by stimulating free fatty acid uptake through increased expression of fatty acid translocase CD36 and intracellular retention of free fatty acid by controlling the expression of fatty acid-binding protein aP2. Expression of perilipin, a predominant protein associated with adipocyte lipid droplets and regulating adipocyte lipid storage (33) is also stimulated by Meda-4 (Table 1). Increased lipid storage in 3T3-L1 adipocyte reflects an imbalance in triglyceride synthesis, lipolysis, fatty acid β-oxidation, and glucose metabolism (1). In accordance with this, the mRNA of glucose transporter-4, acyl-Coenzyme A oxidase 1, fatty acid synthase, hormone-sensitive lipase (HSL), and lipoprotein lipase (LPL) were all up-regulated in Meda-4 transfected cells. Interestingly, many of these genes are prominent in microarray data from FORKO 5-month MAT and dysregulated more than 3-fold, P < 0.05. By using DAVID Gene Functional Classification Tool (http://david.abcc.ncifcrf.gov), these genes are clustered as adipogenesis and lipid metabolism genes (Supplemental Table 2B), supporting their involvement in adipocyte expansion in FORKO mutants.

To further dissect Meda-4 requirement for adipocyte differentiation, we used an additional tool of short hairpin RNA (shRNA) (34, 35) mediated down-regulation of Meda-4 during 3T3-L1 differentiation process. In this series of experiments, two subclones were selected for further study in which Meda-4 expression was down-regulated to 40% and 24% in clones 12 and 17, respectively, as compared with control (Fig. 3G). As indicated, fewer lipid drops accumulate in 3T3L1-shRNA Meda-4 cells after differentiation at d 10 (Fig. 3H). Glucose uptake in 3T3L1-shRNA Meda-4 at d 10 was also significantly reduced with or without insulin stimulation (Fig. 3I). Inhibition of Meda-4 expression also led to profound down-regulation of corresponding key transcriptional factors C/EBP-α, Ppar-γ, Ppar-γ2 known to be required for adipocyte differentiation, but the preadipocyte marker Pref-1 was up-regulated. Other genes involved in lipid accumulation and metabolism were also affected (Table 2). Thus both gain and loss of function studies confirm that Meda-4
is necessary for the processes that promote adipogenesis and lipid accumulation in adipocytes through regulation of transcription factors C/EBP-α and Ppara-γ2 and consequently increase adipogenesis with further involvement in adipocyte lipid and glucose metabolism.

**Meda-4 acts upstream of Ppara-γ during adipogenesis**

The observation that Ppara-γ2 gene expression is compromised in Meda-4-deficient adipocytes suggested that Meda-4 function is required at a time point preceding Ppara-γ2. Therefore, expression patterns of Meda-4 and Ppara-γ2 during 3T3L1 differentiation were examined at different time points shown in Fig 4, A and B. This showed that Ppara-γ2 expression level was very low initially and not induced until 16 h after differentiation. However, in contrast to this, significant expression of Meda-4 was evident at d 0, which then increased further as early as 2 h after the start of differentiation, with a peak at 4–8 h and reduced to a lower level at 24 h, followed again by high levels in the mature adipocyte. Notably, the induction of Meda-4 expression at 2 h preceded the induction of Ppara-γ2 at 16 h, which is consistent with the possibility that Meda-4 could function upstream of Ppara-γ2.

To directly test whether Meda-4 acts upstream of Ppara-γ in adipogenesis, we attempted a rescue experiment in Meda-4-deficient cells using Ppara-γ ligand pioglitazone. In 3T3-L1-Meda-4shRNA cells, lipid content is lower than control cells, supporting that Meda-4 is required for adipogenesis. Pioglitazone treatment increased lipid accumulation in control cells (3T3-L1 vector) as expected, suggesting that activation of Ppara-γ2 effectively promotes adipogenesis when provided with sufficient ligand. Ligand
treatment also restored lipid accumulation in 3T3-L1
Meda-4 shRNA) (Fig 4C). Thus, an alternate mode of
Ppar-γ activation can partly rescue adipogenesis block in
this setting.

### Influence of estrogen on Meda-4 expression in mouse adipocytes in vivo and in vitro

Because Meda-4 was dominant in WAT and specifically
up-regulated in MAT in 5-month-old FORKO females
(that have combined estrogen deficiency and high circu-
lating androgen), direct sex hormonal regulatory influ-
ence(s) is implicated. However, because estrogen treat-
ment in such a chronic setting did not reverse the up-
regulation of Meda-4 expression in the adipose tissue
(data not shown), we examined an alternative experimen-
tal design of acute hormone deficiency. Thus WT female
(data not shown), we examined an alternative experimen-
tal design of acute hormone deficiency. Thus WT female
(groups 2 and 3, circulating sex hormones were eliminated
with prompt atrophy of the highly estrogen-sensitive
uterus. Thus, uterine weight and morphology served as
effective indicators of circulating estrogen (Fig. 5B). In
OVX mice that were treated with vehicle, uterine weight
was reduced by 80%, but Meda-4 in MAT was up-regu-
lated by 40% (P < 0.05). These changes were fully re-
versed by a 10-d treatment with estrogen in group 3 (Fig.
5D) that also completely restored the atrophied uterus
(Fig. 5B). Here the uterine vasculature was similar to
group 1 (Sham), indicating the effectiveness of estrogen
supplementation. However, although obesity and body
weight gain had not yet manifested in this short span in
OVX mice (Fig. 5C), Meda-4 expression was attenuated in
OVX+E2 group, as compared with OVX (Fig. 5D). An in
vitro study using adipocytes further indicated that E2
treatment (20 nm and 2 μM) could also cause down-regu-
lation of endogenous Meda-4 expression in differenti-
ated 3T3-L1 adipocytes (d 8) (Fig. 5E). Time course study
showed that E2 treatment (200 nM) for 8 h and 16 h down-
regulated Meda-4 expression in 3T3-L1 adipocyte (d 8)
(Fig. 5F).

### Discussion

Bulging waist circumference is a cause for concern in the
rising epidemic of obesity and type 2 diabetes in all pop-
ulations and both sexes and is also noted in adolescents.
Increased visceral fat accumulation in postmenopausal
women is also highly deleterious leading to the onset of
insulin resistance and type 2 diabetes (36) as well as rising
cancers (6). Therefore, it is important to shed light on
molecular events surrounding adipogenesis under condi-
tions of hormonal deficiency or imbalances. In this con-
text, our study reporting the cloning and characterization
of a novel gene called Meda-4 from visceral adipose tissue

### TABLE 1. Adipogenesis and differentiation-related gene regulation by Meda-4 overexpression in adipocytes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold change (3T3L1 Meda-4 vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxisome proliferator-activated receptor γ2</td>
<td>Ppar-γ2</td>
<td>2.1a</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor γ</td>
<td>Ppar-γ</td>
<td>1.76b</td>
</tr>
<tr>
<td>CCAAT/enhancer binding protein α</td>
<td>CEBP-α</td>
<td>1.49a</td>
</tr>
<tr>
<td>Adipocyte fatty acid-binding protein 2</td>
<td>aP2</td>
<td>1.36a</td>
</tr>
<tr>
<td>CD36 antigen</td>
<td>CD36</td>
<td>3.52b</td>
</tr>
<tr>
<td>CCAAT/enhancer binding protein δ</td>
<td>CEBP-δ</td>
<td>2.74a</td>
</tr>
<tr>
<td>Glucose transporter type 4</td>
<td>Glut-4</td>
<td>2.56a</td>
</tr>
<tr>
<td>Preadipocyte factor 1</td>
<td>Pref-1</td>
<td>0.25a</td>
</tr>
<tr>
<td>Perilipin 1</td>
<td>Plin-1</td>
<td>1.76a</td>
</tr>
<tr>
<td>Acyl-Coenzyme A oxidase 1</td>
<td>Acyl-oX1</td>
<td>2.86a</td>
</tr>
<tr>
<td>Fatty acid synthase</td>
<td>Fas</td>
<td>2.25a</td>
</tr>
<tr>
<td>Lipoprotein lipase</td>
<td>Lpl</td>
<td>1.55a</td>
</tr>
<tr>
<td>Hormone sensitive lipase</td>
<td>Hsl</td>
<td>2.36a</td>
</tr>
</tbody>
</table>

With gain of function (3T3-L1 Meda-4 vs. control). Cells were differentiated as described in Materials and Methods, and mRNA level of indicated genes were measured on d 8 of differentiation (d 8) by Q-PCR. (n = 4). a, P < 0.05; b, P < 0.01.

### TABLE 2. Adipogenesis and lipid storage-related gene regulation by Meda-4 down-regulation in adipocytes

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold change (3T3L1 shRNA Meda4 vs. control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ppar-γ2</td>
<td>0.18b</td>
</tr>
<tr>
<td>Ppar-γ</td>
<td>0.32a</td>
</tr>
<tr>
<td>CEBP-α</td>
<td>0.02b</td>
</tr>
<tr>
<td>aP2</td>
<td>0.34b</td>
</tr>
<tr>
<td>CD36</td>
<td>0.02b</td>
</tr>
<tr>
<td>Glut-4</td>
<td>0.1a</td>
</tr>
<tr>
<td>Pref-1</td>
<td>2.15b</td>
</tr>
<tr>
<td>Plin-1</td>
<td>0.2a</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>0.05b</td>
</tr>
<tr>
<td>Fas</td>
<td>0.6a</td>
</tr>
</tbody>
</table>

Loss of function (3T3-L1 shRNA Meda-4 vs. control) study. Cells were differentiated after treatment, and mRNA level of indicated genes were measured on d 10 (differentiation d) by Q-PCR. Results are expressed after normalization to β-actin. (n = 4). a, P < 0.05; b, P < 0.01.
in both obese mutant female mice and women assumes significance. *Meda-4* up-regulation in obese condition in FORK0 female mutants suggests close links of *Meda-4* expression and sex hormonal imbalance-induced obesity progression and metabolic alterations (12). *Meda-4* is expressed predominantly in the WAT being higher in the AF (Fig. 2, A, B, and D), suggesting adipocyte as an important site and target of *Meda-4* function. Because it is not a secreted protein, it is likely to have an intracrine role. We validated this hypothesis by employing complementary experimental gene overexpression and knockdown approaches examining several aspects of adipocyte cell biology in vitro. Both manipulations indicated *Meda-4* involvement in processes that promote adipocyte differentiation, lipid accumulation, and glucose uptake in mature adipocytes. It clearly affects two major transcription regulators, Ppar-γ2 and C/EBP-α, and target genes involved in adipocyte differentiation and lipid storage. Our observation of highest *Meda-4* expression in the adipose tissue (Fig. 1, C and D) suggests that this gene could exert its predominant effect at this site to participate in adipocyte expansion although alternative functions in other tissues cannot be ruled out at this time. In this regard, understanding the sexual dichotomy in *Meda-4* expression seen in the heart, brain, and pancreas (Fig. 1, C and D) requires more detailed studies.

Adipocyte differentiation is a complex developmental process that involves the coordinated interplay of numerous factors. For the first time, we now demonstrate that a new player *Meda-4* can also promote adipogenesis by increasing adipocyte differentiation. At a molecular level, we suggest that *Meda-4* is an upstream regulator of transcription factor Ppar-γ2, as supported by both gain and loss of function studies; earlier induction of *Meda-4* than Ppar-γ2 expression by time course study (Fig. 4, A and B); rescue of lipid accumulation by Ppar-γ ligand in *Meda-4*
knockdown cells (Fig 4C). Of the two Ppar-γ isoforms, Ppar-γ1 and Ppar-γ2, Ppar-γ1 is ubiquitously expressed whereas expression of Ppar-γ2, the splice variant, is restricted to adipose tissue. Ppar-γ2 is reportedly the more adipogenic Ppar-γ isoform in vitro (37). Accordingly shRNA-Meda-4 down-regulation of Ppar-γ2 to a greater extent than Ppar-γ in our experimental setting is fully consistent with these observations. Ppar-γ has been extensively studied as a key regulator in development and maintenance of the adipose tissue. Furthermore, synthetic Ppar-γ agonists are also used in type 2 diabetes treatment (33). Our study found a close relationship of Meda-4 with Ppar-γ/γ2 at both the phenotypic and molecular levels: both genes promote preadipocyte differentiation, lipid storage, and glucose uptake in adipocytes. Many Meda-4-regulated genes (Table 1) related with lipid storage and metabolism are also regulated by Ppar-γ (33, 38). The induction of Meda-4 expression early at 2 h of 3T3-L1 adipocyte differentiation is consistent with a role for Meda-4 in determining subsequent events in adipogenesis, promoting factors Ppar-γ and C/EBP α. Previous studies have indicated that failure of any of these events is sufficient to prevent normal adipocyte differentiation (39, 40).

The expression profile of Meda-4 during the early stages of adipocyte differentiation is nearly identical to that of C/EBP β and C/EBP δ, which is induced at high levels at 10 h after stimulation with differentiation mixture followed by a sharp decline before the onset of Ppar-γ and C/EBP α (41). The expression of C/EBP δ responding to methylisobutylxanthine induction of the cAMP-signaling pathway is also known (42). Because both mouse and human MEDA-4 proteins are predicted to contain domain for cAMP- and cGMP-dependent protein kinase phosphorylation (Supplemental Table 3), it is possible that Meda-4 may also share a similar regulatory mechanism that needs to be verified in future investigations. Interestingly, perusal of the human genome database indicates that human MEDA-4 is located in close proximity to several other genes known to be related to lipid and glucose metabolism and involved in diseases of insulin resistance, hypertension, or diabetes. Thus examples to note are arachidonate 5-lipoxygenase-activating protein (ALOX5AP) (43); Klotho (44); pancreatic and duodenal homeobox 1 (45).

Menopausal transition (low estrogen/androgen ratio) increases the rates of obesity and metabolic syndrome during aging in women (46). Hormonal replacement therapy is known to reduce visceral obesity in postmenopausal women (47) but has other potentially adverse effects. We have previously shown that FORKO female mice experiencing early obesity exhibit age-related mimicry of metabolic syndrome (18). In FORKO females, abdominal adiposity is associated with induction of visceral inflammation and activation of novel cytokine pathways (21). In line with this, we investigated estrogen regulation of Meda-4 expression in MAT. Clearly, low estrogen favors adipose tissue up-regulation of Meda-4 in both FORKO (Fig. 1A) and acutely OVX females (Fig. 5D). Conversely Meda-4 expression is also down-regulated by E2 treatment in 3T3-L1 cells and in MAT of acutely estrogen-deficient OVX (Fig. 5, D–F). However, in chronically estrogen-deficient FORKO females at 5 months, our current E2 treatment modality was insufficient to attenuate high Meda-4 expression. We speculate that the difference could be due, in part, to the duration of estrogen deficiency and timing of treatment could play a role in reversing adversities. In fact, other studies in human subjects have indicated that timing issues are critical to derive useful benefits from hormone replacement after menopause (48). The persistently higher androgen level in FORKO females, as opposed to low androgen level in acute OVX females, could also play a role.

Based on studies in 3T3-L1 cells, we find that Meda-4 gene functions as one of the positive effectors to promote adipogenesis and triglyceride storage in adipocytes. It also promotes glucose uptake in adipocytes when cells remains insulin sensitive. In FORKO mice, visceral fat accumulation increased at both 5 month and 9 months; Meda-4 expression also increased at both ages, supporting the notion that Meda-4 promotes adipogenesis and glucose homeostasis. However, FORKO females progressively developed insulin resistance starting at 9 months of age. We attribute this apparent discordance to the impact of a dysfunctional deep visceral fat, causing other negative factors including proinflammatory gene(s) to be up-regulated, including the novel gene MEDA-7 (21), and aberration of adiponectin signaling and thus insulin signaling are eventually impaired in MAT (18). At this stage of impending disease, the up-regulation of Meda-4 apparently failed to compensate the cumulative effect of negative events evident at 9 months of age in these mutants.

In conclusion, our study reports mouse and human Meda-4 as a newly identified molecular entity that contributes to adipocyte expansion and metabolism in states of hormonal imbalance in females associated with obesity and metabolic syndrome. Expression of MEDA-4 in morbibly obese individuals in a fat depot-specific manner and high homology of mouse and human MEDA-4 proteins suggest conserved function(s) across species. To address its potential role in human obesity and metabolic disorders, further in-depth investigations in relation to gender and age are required. The chromosomal location of human MEDA-4 in the vicinity of several genes already known to affect lipid and glucose metabolism and in-
volved in atherosclerosis or diabetes (43–45) is also noteworthy for its potential clinical relevance that warrants additional scrutiny.

Acknowledgments

We thank our colleagues Drs. Pierre Leclerc (Hotel-Dieu Hospital) and Remi Rabassa-Lhoret and May Faraj (at the Clinical Research Institute of Montréal) for their help in securing fat tissue samples from patients. We are also grateful to Dr. J. P. Aravindakshan (Clinical Research Institute of Montreal) for help in the initial microarray studies and Ms. Lilliane Chamas (Clinical Research Institute of Montreal) for cell culture work.

Address all correspondence and requests for reprints to: M. R. Sairam, Ph.D., Molecular Endocrinology Laboratory, Clinical Research Institute of Montréal, 110 Pine Avenue West, Montréal, Québec, Canada H2W 1R7. E-mail: sairamm@ircm.qc.ca

This work was supported in part by grants from the JNJ (New Brunswick, NJ) focused giving program and Canadian Institutes of Health Research.

Disclosure Summary: No potential conflicts of interest relevant to this article are reported by the authors.

References

30. Zhou YT, Wang ZW, Higa M, Newgard CB, Unger RH 1999 Re-
44. Wang Y, Sun Z 2009 Klotho Gene delivery prevents the progression of spontaneous hypertension and renal damage. Hypertension 54:810–817

Take advantage of the Endocrine Society’s online ABIM approved Maintenance of Certification (MOC) self-assessment resources.