CNS Neuroscience & Therapeutics

ORIGINAL ARTICLE

CNS Neuroscience & Therapeutics

Subfatin is a Novel Adipokine and Unlike Meteorin in Adipose and Brain Expression

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Keywords
Adipokines; Brain; Caloric restriction; Meteorin; Subfatin.

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Received 5 November 2013; revision 25 November 2013; accepted 29 November 2013

doi: 10.1111/cns.12219

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SUMMARY

Aims: Adipose tissue releases adipokines that play important roles in metabolic and cardio-cerebro-vascular homeostasis. This study was to discover novel adipokines using caloric restriction model. **Methods:** Adipokine candidates were captured by gene array and bioinformatics analysis and verified by preparation of recombinant protein and antibody. **Results:** We established a potential secreted protein database containing 208 genes and identified a novel adipokine, Subfatin, that was the highest expressed in subcutaneous fat of both rodents and humans among 15 detected tissues. The secreted mammalian Subfatin was a glycosylated protein. Subfatin was located diffusely throughout the adipose tissue except lipid droplets, with comparable expression between adipocytes and stromal cells, but much lower expression in macrophages than adipocytes. Subfatin was downregulated in white adipose tissue of caloric restriction rats, whereas dramatically upregulated during white adipocyte differentiation as well as in white adipose tissue of diet-induced obese mice. Subfatin was annotated as Meteorin-like (Metrnl) in public databases, a similar transcript of Meteorin (Metrn, also known as glial cell differentiation regulator). Meteorin displayed a brain-specific expression and was scarce in various adipose tissues, in contrast to the tissue expression patterns of Subfatin. **Conclusions:** Subfatin is a novel adipokine regulated by adipogenesis and obesity, with tissue distribution different from its homologue Meteorin.

Introduction

Adipose tissue is a highly active endocrine organ that can produce and secrete various proteins/peptides termed adipokines [1–3]. Adipokines are involved in the regulation of multiple physiological functions, including metabolic homeostasis, insulin sensitivity, cardio-cerebro-vascular function, immunity, and inflammation [1–3]. Accumulating evidence indicates that the link between obesity and cardio-cerebro-vascular disease is adipose dysfunction, in which an imbalance between beneficial and harmful adipokines plays an important role [1–3].

Many adipokine studies contribute to a new emerging field of "adipose-brain axis" research. It is originally attributable to a breakthrough for the discovery of leptin that signals to the brain the state of energy stores in peripheral tissues and then controls energy balance and body weight [4]. Our works on identification of visfatin (also known as NAMPT) as a target for stroke therapy provide new insights into understanding of adipose-brain axis [5–9]. In addition, we demonstrate a novel leptin receptor signaling in the brain for control of body weight under caloric restriction, suggesting FTO may be a therapeutic target for leptin resistance obesity [10]. We also studied the adipokines in adipose renin-angiotensin system [3] and the inflammatory adipokines such as TNFα [11]. We gave much attention to all known adipokines for their roles in cardio-cerebro-vascular physiology, pathology, and pharmacology [2,3,12]. It appears more than 100 adipokines have been reported. In adipokine world, in addition to further investigation on the known adipokines, a renewed effort should be made toward the discovery of novel adipokines, because it is likely that far more exist than have currently been identified [2,13].

This study aimed for the discovery of totally novel adipokines. As reported in the past two decades, classically, most adipokines were identified using obesity models [14–17]. Secretome analysis was used in recent researches, such as obese adipose secretome [18], adipogenesis secretome [19], and adipose depot-specific secretome [20]. We sought to search new adipokines using caloric restriction model that has never been used in the discovery of adipokines previously. A large number of studies from our and other groups have shown that caloric restriction can produce various benefits on whole-body health, such as control of body weight, delay of age-related metabolic and cardio-cerebro-vascular diseases, and extension of life span [1,10,21,22]. We considered that application of the caloric restriction intervention

strategy would identify new adipokines and might help to reveal new mechanisms underlying caloric restriction benefits.

In 2007, we performed 18 weeks of caloric restriction in adult rats and completed gene array detection of three adipose depots. In early 2008, 12 chips of whole-transcriptome gene expression data were analyzed [3], and bioinformatics methods were used to extract all the genes speculated for encoding the secreted proteins. Among these potential genes, a gene annotated as "Meteorin-like" was very highly expressed in subcutaneous adipose tissue without any research report on its expression and function in PubMed. This gene was therefore screened as the only candidate for our further identification.

Meteorin-like (Metrnl) is a similar transcript of Meteorin (Metrn). The name Meteorin is a vivid description for its function transforming glial cells into cells with an elongated tail, looking like meteors [23], and therefore Meteorin is also known as glial cell differentiation regulator. Meteorin exhibits a brain-specific expression [23,24]. Meteorin-like is named after Meteorin, according to their sequence similarity. However, little is known for this similar transcript of Meteorin. Our early preliminary experiments show its expression level is low in the rodent brain, indicating Meteorin-like is unlike Meteorin in the brain expression. This study identified this similar transcript of Meteorin as a novel adipokine highly enriched in subcutaneous fat, while Meteorin was scarce in adipose tissue. Therefore, we propose a more appropriate name "Subfatin" (refers to subcutaneous fat highly expressed protein) instead of "Meteorin-like" in our research.

Materials and methods

Male Sprague-Dawley rats and mice were purchased from Sino-British SIPPR/BK Laboratory Animal Ltd, Shanghai, China. Animals were housed under controlled temperature (23–25°C) and lighting (12-h light-dark cycle, lights from 08:00 to 20:00 h) with free access to tap water and standard chow [25], unless otherwise noted. Animals were accommodated for 1–3 weeks before experiments. All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the ethical committee for animal experiments of the Second Military Medical University.

Human tissues were collected from three male patients for gastric cancer operation using a protocol approved by the ethical committee of Changhai Hospital of Shanghai. All patients were given a detailed description of the study, and their written informed consent was obtained. Samples were preserved in liquid nitrogen before RNA isolation.

Caloric Restriction in Rats

Caloric restriction (CR) was performed in adult rats for 18 weeks. Briefly, at 9–10 weeks of age, rats were divided into two groups: *ad libitum* (AL) and CR. AL animals were allowed unlimited access to standard chow, whereas the CR animals were restricted to 60% of the food intake consumed by AL animals, as described in our previous reports [10,22]. After 18 weeks of CR treatment, animals were fasted overnight (approximately 12 h) and anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Blood was collected from the inferior vena cava for the determination of serum parameters. Perivascular adipose tissue (PVAT) of descending thoracic aorta, subcutaneous adipose tissue (SAT) of inguinal region, and mesenteric adipose tissue (MAT) were obtained for gene expression profiling analysis [3,8].

Gene Array Analysis

Gene expression profiling was performed using the Illumina Sentrix Rat Ref-12 Expression BeadChip platform, as described elsewhere [26]. Briefly, total RNA of different fat depots was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified with the RNAeasy mini kit (Qiagen, Hilden, Germany). The integrity was assessed using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Then, 1 μg RNA was used for cDNA synthesis and, cDNA was hybridized to the RatRef-12 expression BeadChip. The hybridized array was scanned with a BeadArray Reader (Illumina, San Diego, CA, USA).

Screening of New Adipokine Candidates

Genes common to both gene array and Secreted Protein Database (http://spd.cbi.pku.edu.cn/) were extracted and then selected with AVG_signal > 2500 in gene array. To avoid omission of canonical secreted protein, genes with AVG_signal > 2500 in gene array were all filtered with SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) for searching signal peptide and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) for excluding transmembrane protein. A secreted protein database with high gene expression in rat adipose was established by above selections. All the selected genes were searched in PubMed for new adipokine candidates. Subfatin was finally screened as the only candidate without any report on its expression or function in early 2008.

Preparation of Polyclonal Antibodies Against Subfatin

The Subfatin open reading frame was cloned from the mouse brain tissue, fused with 6 × His tag at C-terminal, inserted into pET21 vector (Figure S1), and transformed into competent DH5α cells. Subfatin-His_6 recombinant protein was purified through nickel-affinity chromatography column (Qiagen), as described in our previous report [9]. Rabbits were injected with Subfatin-His_6 recombinant protein. Antibodies were affinity-purified on an antigen column.

Preparation of Recombinant Proteins in Mammalian Cells

The Subfatin open reading frame with a C-terminal-fused His_6 tag was inserted into eukaryotic expression vector pCI-neo (pCI-neo-Subfatin-His_6) and transfected into HEK-293 or COS-7 cells (Figure S2). The transfected cells were cultured for 60 h in Opti-MEM (Gibco, Paisley, Scotland), after which the Subfatin-His_6 fusion protein was purified from the culture medium with Talon metal affinity resin (Clontech, Basingstoke, UK). The purified protein was desalted and concentrated with a centrifugal filter device (Millipore, Bedford, MA, USA) and identified

by Western blotting with anti-Subfatin and anti-His_6 antibodies or by mass spectrometry.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (PCR) was carried out as described in our previous reports [5,6,27]. Briefly, total RNA was extracted from various tissues or cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A total of 1.5 μg of RNA was used for reverse transcription. Quantitative PCR was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA) and was executed using the SYBR Green PCR master mix (Applied Biosystems) with 1 μL cDNA template in a 20 μL final reaction mixture (95°C for 15 min; 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds, 40 cycles). The average threshold cycle (Ct) for each gene was determined from triplicate reactions, and the target gene expression was normalized to GAPDH. Primer sequences were shown in Table S1.

Culture and Differentiation of 3T3-L1 Preadipocytes

3T3-L1 preadipocytes were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco), as described in our previous report [11]. For differentiation, cells were cultured for additional 2 days after confluence, which was counted as day 0, and cultured in differentiation medium [DMEM containing 10% FBS, 1 μg/mL insulin (Sigma, St. Louis, MO, USA), 0.1 μg/mL dexamethasone (Sigma), and 112 μg/mL isobutylmethylxanthine (IBMX, Sigma)]. After 2 days, cells were cultured in DMEM containing 10% FBS and 1 μg/mL insulin for 6 days. The culture medium was changed every other day.

Western Blotting

Western blotting was carried out as described in our previous reports [6,21,28]. Briefly, total protein was extracted using cell lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixtures]. Equal amounts of protein were loaded and separated via 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to 0.4-mm nitrocellulose membranes, and the membranes were blocked in 5% skim milk for 3 h before incubating with antibody (1:1000) diluted in blocking solution overnight. After washing three times with Tris-buffered saline with Tween 20, fluorescein-labeled secondary antibodies were incubated with the membranes for 1 h at room temperature. Blots were scanned with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Immunohistochemistry

To detect Subfatin expression via immunohistochemistry in adipose tissue, isolated mouse SAT was fixed in 4% paraformaldehyde overnight, dehydrated, hyalinized, paraffin embedded, and sectioned at 5 μm thickness. The tissue slices were hydrated, and antigen retrieval was performed by treating the slides in citrate buffer in a microwave for 15 min. Endogenous peroxidases were inactivated by treating the tissue with 1% H_2O_2 for 20 min. The sections were then blocked with 3% bovine serum albumin in PBS for 1 h and incubated with Subfatin primary antibody (Sigma, 1:100) at 4°C overnight, sealed with antibody dilution (1:100), incubated with avidin-labeled secondary antibody at room temperature for 1 h, reacted with streptavidin–biotin complex for 30 min, and the color reaction was developed using 3,3′-diaminobenzidine.

High-Fat Diet in Mice

Diet-induced obesity mouse model was created as we described elsewhere [11]. Obesity was induced by a high-fat diet containing about 60% of calories from lipids (D12492; Research Diets, New Brunswick, NJ, USA) when the mice were 6 weeks old and the control mice were fed with a standard chow diet (Sino–British SIPPR/BK Lab Animal Ltd.). After 12 weeks feeding, mice were sacrificed under anesthesia, and subcutaneous adipose tissue samples were harvested to examine the expression of Subfatin by quantitative PCR.

Statistical Analysis

Data were presented as means ± SE. Differences between groups were examined using one-way analysis of variance (ANOVA). Statistical significance was judged at $P < 0.05$.

Results

Food Intake, Body Weight, and Serum Parameters in CR Rats

The food intake in CR rats was adjusted every week and restricted to 60% of food consumption in AL rats last week, which was shown in Figure 1A. During 18 weeks of CR treatment, body weight was much lower in CR rats than in AL rats (Figure 1B). At the end of CR treatment, body weight gain was much less in CR rats (35 ± 12 g) than in AL rats (223 ± 6 g). These further indicate that CR controls body weight efficiently. Serum parameters, including glucose, insulin, triglycerides, total cholesterol, LDL cholesterol, and HDL cholesterol, demonstrated a slight reduction but no significant difference in CR rats compared with AL rats (Figure 1C).

Subfatin is Screened as a Novel Adipokine Candidate

Of the ~22,500 genes in the gene array, 1227 genes existed in the Secreted Protein Database. Among these 1227 genes, 205 genes were highly expressed in the gene array with AVG_signal > 2500 (Figure 2A). Meanwhile, all the genes with AVG_signal > 2500 in the gene array were filtered with SignalP and TMHMM Servers, and 108 genes were selected for encoding canonical secreted proteins (Figure 2A). After merging both gene subsets screened by the two selection approaches, 208 genes were totally extracted, constituting a secreted protein database with high gene expression in rat adipose. After searching these

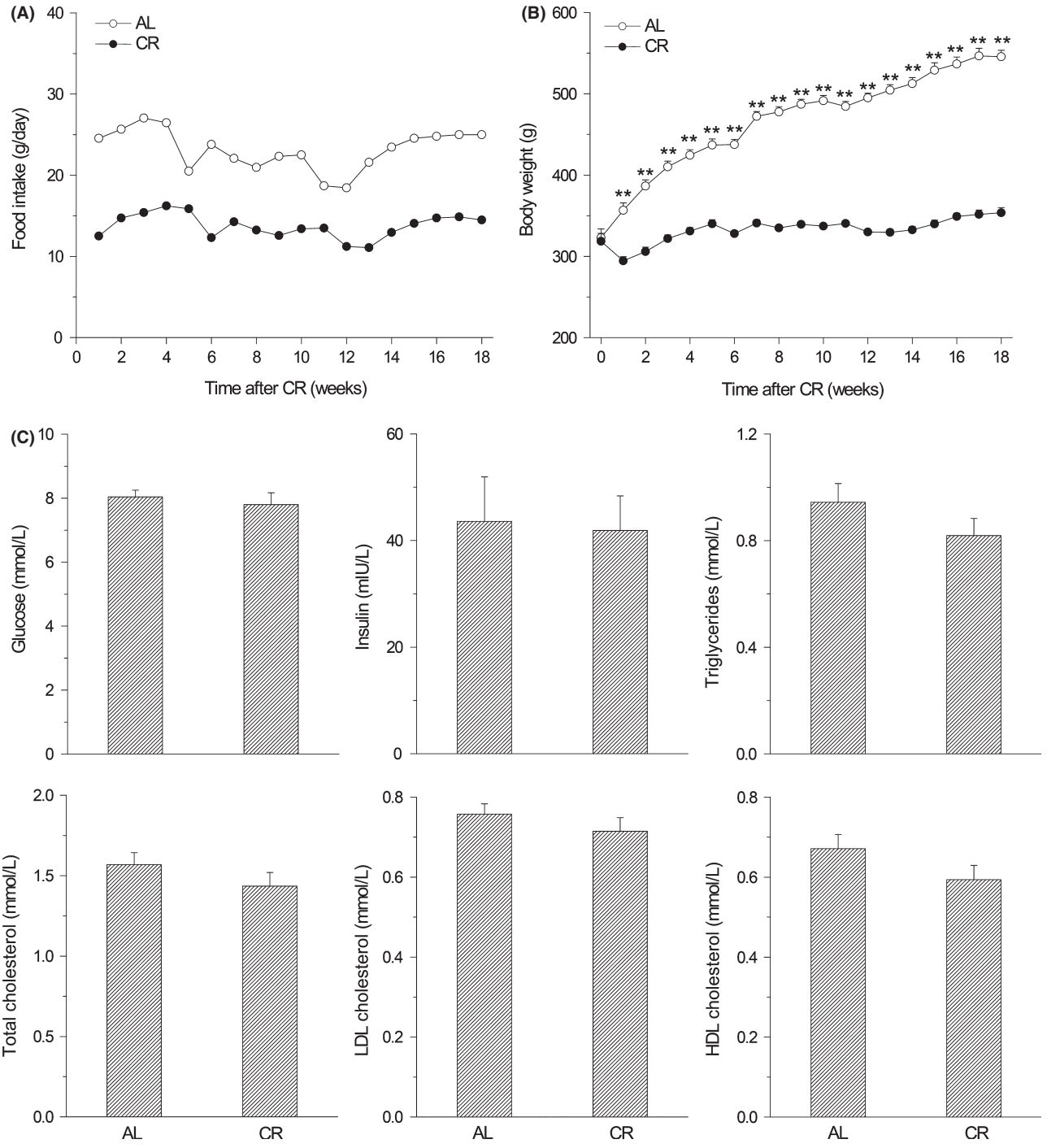

Figure 1 Food intake, body weight, and serum parameters in *ad libitum* (AL) and caloric restriction (CR) rats. (**A**) Food consumption of AL and CR rats. (**B**) Body weight curve of AL and CR rats. $^{**}P < 0.01$ versus AL. (**C**) Serum glucose, insulin, triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol in AL and CR rats. n = 8 in each group.

208 genes in PubMed, Subfatin was finally selected as the only candidate without any dedicated research on its expression or function in early 2008 (Figure 2A).

The NCBI Gene ID for the rat, mouse, and human Subfatin was 316842, 210029, and 284207, respectively. The open reading frame for this gene contained 936 bp, encoding 311 amino acids (aa). Bioinformatics analysis of Subfatin protein sequence indicated a signal peptide containing 45 aa in the NH_2-terminal segment (Figure 2B) and no transmembrane region (Figure 2C). A total of 239 amino acids (77%) were identical in the

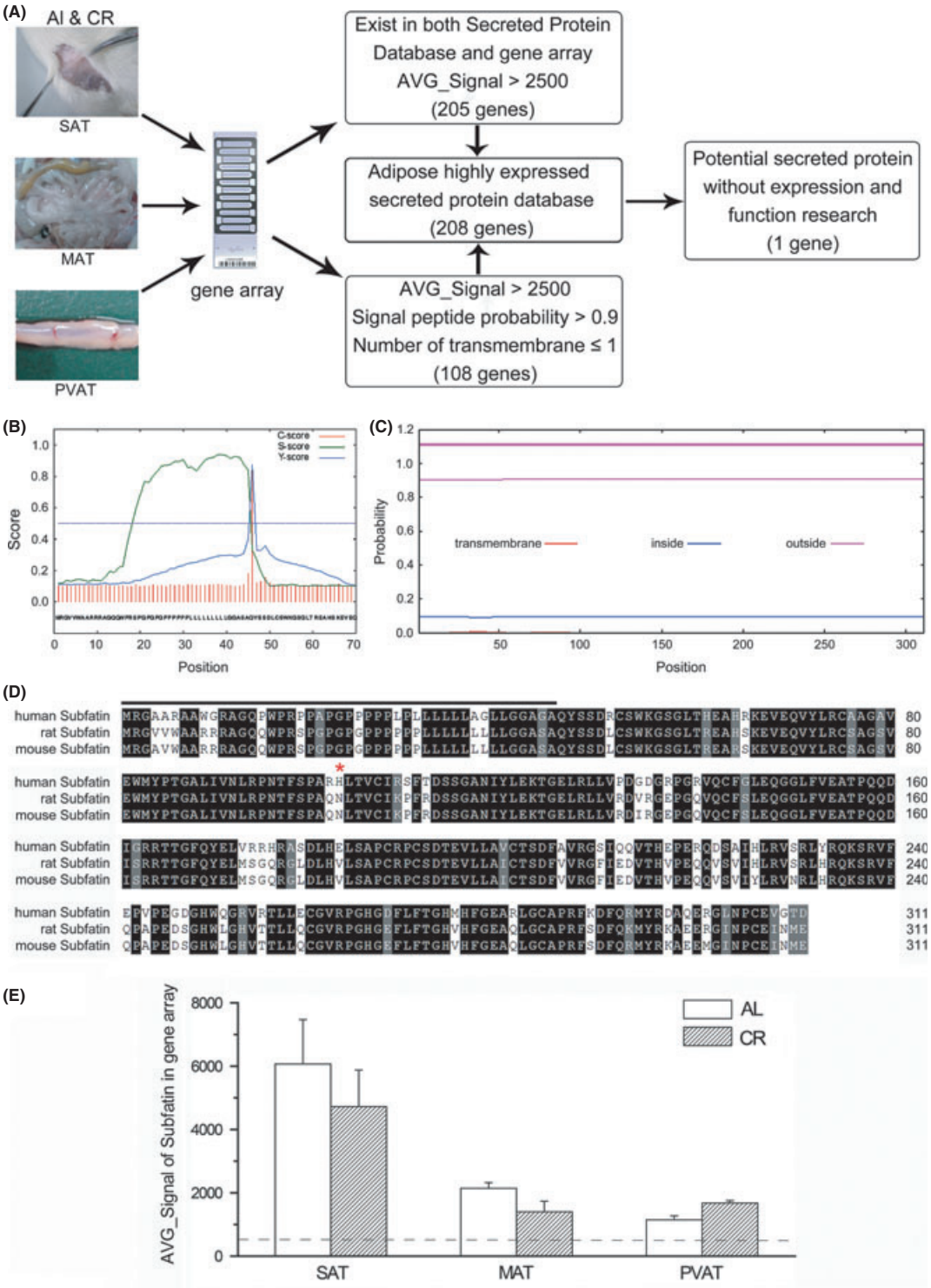

Figure 2 Screening and identification of Subfatin from adipose tissues. (**A**) Flowchart screening process of novel adipokines. (**B**) Graphical output from SignalP Server showing a 45 aa signal peptide in Subfatin. (**C**) Graphical output from TMHMM Server showing no transmembrane domain in Subfatin. (**D**) Amino acid alignment of human, rat, and mouse Subfatin sequences. Residues identical in all proteins were marked in black boxes, and similarity was shown in gray boxes. The putative NH2-terminal signal sequences were indicated by the solid overline, and potential glycosylation site in mouse and rat Subfatin was indicated by red star. (**E**) The AVG_signal of Subfatin in various adipose tissues under CR. The mean value of global gene expression profiles was indicated by dotted line. AL, *ad libitum*; CR, caloric restriction; SAT, subcutaneous adipose tissue; MAT, mesenteric adipose tissue; PVAT, perivascular adipose tissue.

human, rat, and mouse proteins. And there existed a potential glycosylation site in the mouse and rat Subfatin at aa 103 (Figure 2D). Subfatin and Meteorin exhibit approximately 40% amino acid identity to each other and comprised a new two-member protein family without known conserved domains. As shown in Figure 2E, Subfatin expression was abundant in rat adipose gene array especially in SAT (SAT > MAT > PVAT) and was regulated by CR. The mean value of global gene expression profiles was about 500, indicated by dotted line in Figure 2E. Further, the changes in Subfatin expression in different adipose depots after CR were verified with quantitative PCR (Figure S3).

Subfatin is Identified as a Glycosylated Adipokine

The open reading frame for the gene of mouse Subfatin was cloned from the brain tissue (Figure 3A), and Subfatin-His_6 prokaryotic recombinant protein with a molecular weight of ~30 kDa was produced (Figure 3B). A rabbit polyclonal antibody was prepared and verified with Subfatin full-length prokaryotic cell recombinant protein (rSubfatin, Figure 3C). For selecting the appropriate cell line to produce Subfatin mammalian recombinant protein, the Subfatin protein was examined in both cell lysate and serum-free culture medium of Subfatin-

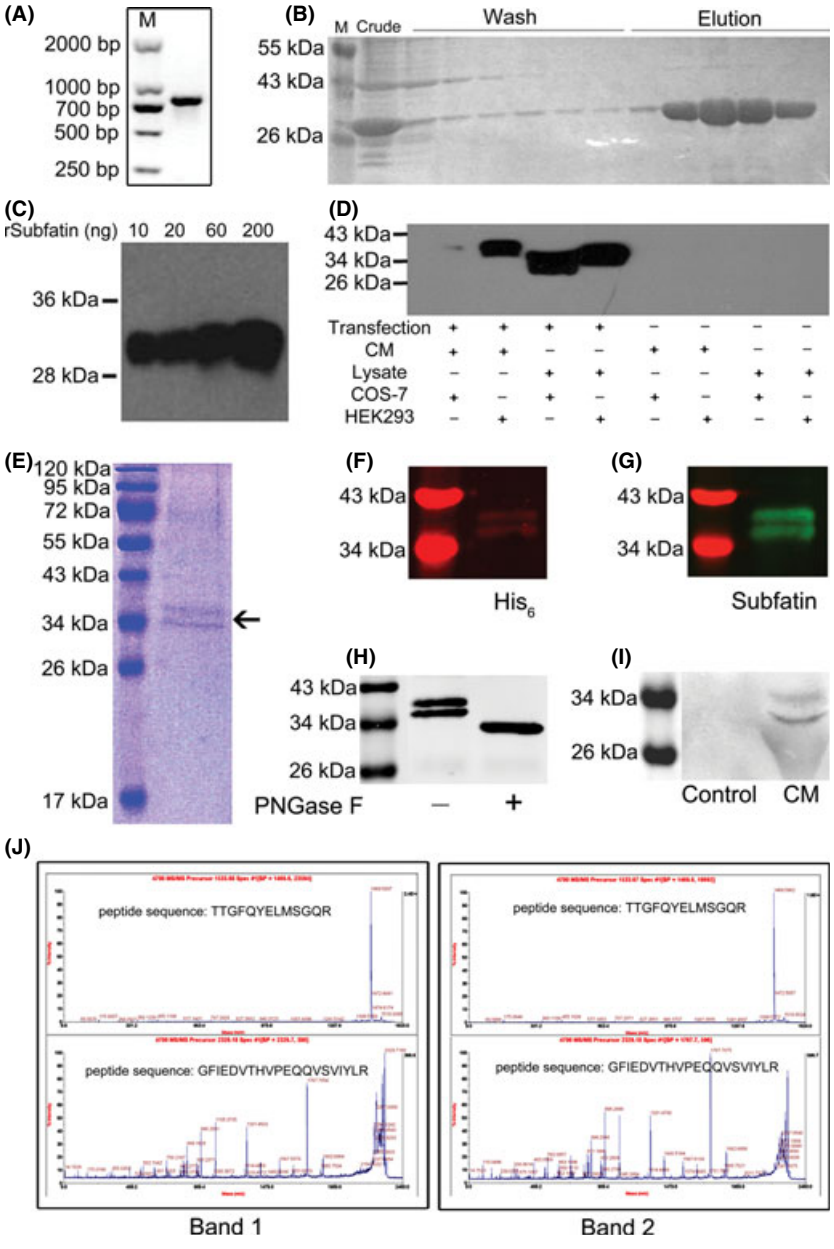

Figure 3 Identification of Subfatin as a novel adipokine. (**A**) Cloning of Subfatin open reading frame. (**B**) The purification of Subfatin prokaryotic recombinant protein. (**C**) Verification of Subfatin antibody using Subfatin recombinant proteins with Western blotting. (**D**) Subfatin was detected in both culture medium and cell lysate of Subfatin-transfected HEK-293 and COS-7 cells. (**E**) Purification of Subfatin recombinant protein from the serum-free culture medium of transfected HEK-293 cells. (**F**) and (**G**) Identification of Subfatin mammalian recombinant protein with anti-Subfatin or anti-His_6 antibody. (**H**) Subfatin mammalian recombinant protein was detected with Western blotting after PNGase F digestion. (**I**) Subfatin in serum-free incubation medium of adipose tissue was detected with Western blotting. (**J**) Identification of Subfatin mammalian recombinant proteins with mass spectrometry. CM, culture medium; PNGase F, glycopeptidase F.

transfected HEK-293 and COS-7 cells. Although Subfatin in cell lysate of HEK-293, with a higher molecular weight, was comparable with that of COS-7 cell lysate, Subfatin in culture medium of HEK-293 was more abundant (Figure 3D). Thus, HEK-293 was used to produce Subfatin recombinant protein. The Subfatin-His_6 fusion protein was purified by affinity chromatography and separated by SDS-PAGE (Figure 3E). After coomassie brilliant blue staining, two bands appeared between 34 and 43 kDa and both bands were recognized by anti-Subfatin and anti-His_6 antibodies (Figure 3F, G). Further, these two bands were both identified as Subfatin protein by mass spectrometry (Figure 3J and Figure S4).

The calculated molecular weight of Subfatin without signal peptide was ~30 kDa, much smaller than that of the mammalian Subfatin recombinant protein we produced. Mouse Subfatin was thought as a potential glycoprotein with a glycosylation site at aa 103. So the difference in molecular weight might be the consequence of glycosylation. Treatment of the recombinant protein with glycopeptidase F (PNGase F) resulted in the collapse of the two bands into a single band of lower molecular weight (~33 kDa), suggesting that the two original bands represent differentially glycosylated forms of Subfatin (Figure 3H). To find out whether adipose tissue can secrete Subfatin, adipose tissue was incubated in DMEM culture medium without serum overnight. Two bands were detected by anti-Subfatin antibody in the incubation medium with the molecular mass between 26 and 34 kDa (Figure 3I).

Subfatin is Highly Expressed in Subcutaneous White Adipose Tissue

To clarify the tissue expression pattern of Subfatin, mouse and human tissues including liver, spleen, muscle, heart, omental adipose tissue (OAT), and SAT were examined by quantitative PCR. Consistent with the data of gene array, Subfatin mRNA expression in SAT was the highest among detected tissues both in mice (Figure 4A) and human (Figure 4B). To further clarify Subfatin expression levels in white adipose tissue and brown adipose tissue, we compared the expression of Subfatin in subcutaneous adipose tissue (white adipose tissue) and interscapular adipose tissue (IAT, a typical depot of brown adipose tissue). As shown in Figure 4C, Subfatin was much higher expressed in subcutaneous white adipose tissue. These are consistent with the expression patterns in rat white (SAT) and brown (PVAT) adipose tissue in the gene array (Figure 2E).

To demonstrate which cell types are Subfatin mainly expressed in, adipocytes and stromal cells were separated by collagenase digestion of fat tissue. No significant difference in Subfatin expression was observed between adipocytes and stromal cells

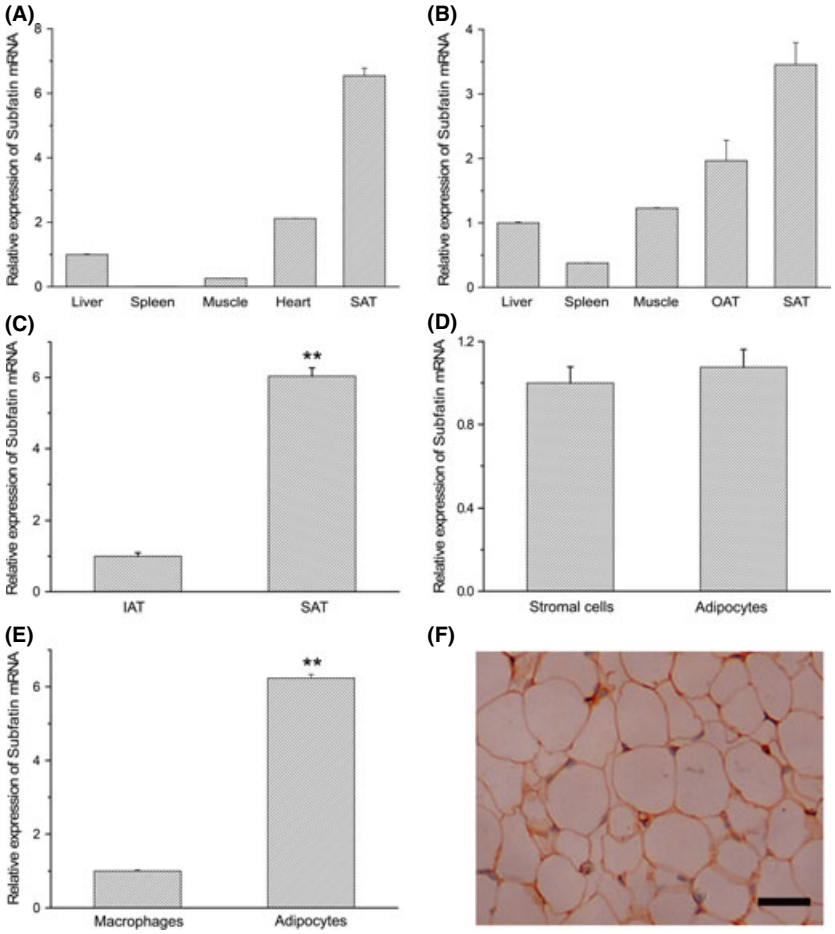

Figure 4 The expression of Subfatin in different tissues and cell types. (**A**) The expression of Subfatin in different tissues of mice (n = 3). (**B**) The expression of Subfatin in different tissues of human (n = 3). (**C**) Comparison of Subfatin expression between subcutaneous adipose tissue (SAT) and interscapular adipose tissue (IAT) of mice (n = 3). $^{**}P < 0.01$ versus IAT. (**D**) Comparison of Subfatin expression between adipocytes and stromal cells isolated from the adipose tissue of mice (n = 4). (**E**) Comparison of Subfatin expression between 3T3-L1 adipocytes and RAW 264.7 macrophage cells. $^{**}P < 0.01$ versus macrophages. (**F**) Subfatin in mouse subcutaneous adipose tissue was detected by immunohistochemistry. Bar = 100 μm.

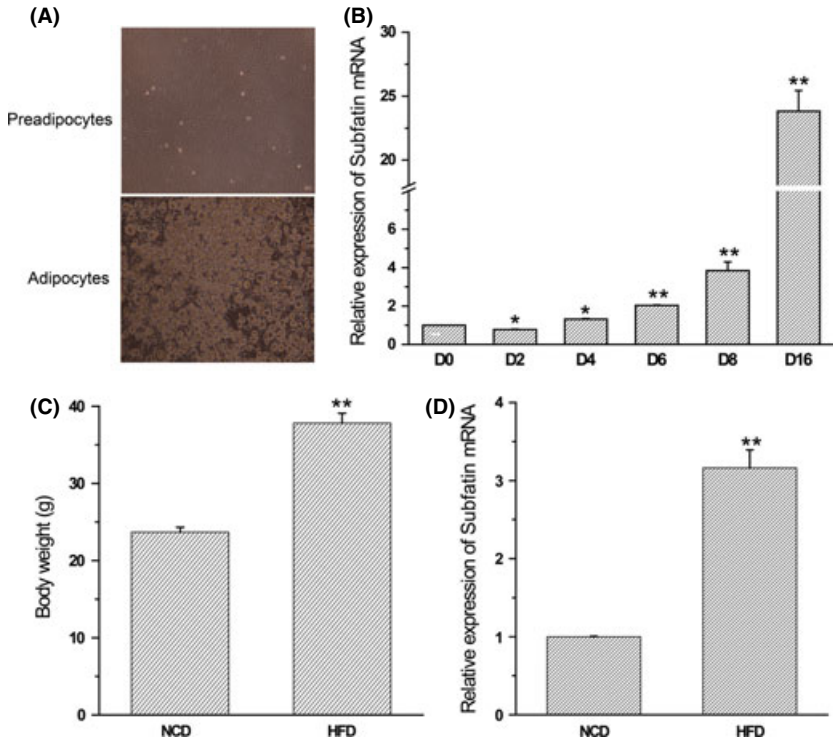

Figure 5 Subfatin expression is regulated by adipogenesis and obesity. (**A**) Representative phase-contrast images of 3T3-L1 preadipocytes and differentiative adipocytes. Bar = 100 μm. (**B**) Subfatin expression during adipocyte differentiation (n = 3). $*P < 0.05$, $**P < 0.01$ versus D0. (**C**) Body weight of diet-induced obesity mice (n = 4). $**P < 0.01$ versus NCD. (**D**) Subfatin expression in subcutaneous adipose tissue of diet-induced obesity mice (n = 4). NCD, normal chow diet; HFD, high-fat diet. $**P < 0.01$ versus NCD.

(Figure 4D). Macrophages exist in stromal cells isolated from fat tissue. We compared the expression of Subfatin between RAW264.7 macrophages and 3T3-L1 adipocytes. Subfatin expression was about sixfolds lower in macrophages than that in adipocytes (Figure 4E). Further, Subfatin expression and location in fat tissue were determined by immunohistochemistry. The results showed that Subfatin exhibited a diffuse distribution throughout the adipose tissue except the lipid droplets (Figure 4F and Figure S5), consistent with its secretion.

Subfatin Expression is Upregulated During Adipogenesis and Obesity

The changes in Subfatin expression during adipocyte differentiation were examined (Figure 5A). Subfatin expression was slightly but statistically significantly decreased at day 2 (induced by IBMX, insulin, and dexamethasone for 2 days) and then increased steadily and dramatically up to ~23 folds at day 16 of differentiation (Figure 5B). To explore Subfatin expression in obesity, diet-induced obesity model was prepared in mice fed a high-fat diet for 12 weeks (Figure 5C). As shown in Figure 5D, Subfatin was higher expressed in adipose tissue of obese mice compared with normal controls.

Subfatin is Unlike Meteorin in Adipose and Brain Expression

To compare the tissue expression patterns between Subfatin and its homologue Meteorin, different parts of brain and adipose tissue together with spleen and thymus were obtained from mice and detected by quantitative PCR. Consistent with the gene array data, Subfatin was highly expressed in adipose tissue especially in SAT (SAT > MAT > PVAT), with much lower expression in three parts of brain and undetected expression in spleen and thymus (Figure 6A). In contrast, Meteorin was highly expressed in the brain especially in forebrain rather than adipose tissue (Figure 6B).

Discussion

Using gene array and bioinformatics analysis in three adipose depots of AL and CR rats, Subfatin was selected as the only novel adipokine candidate, with high expression in adipose tissue especially in SAT and without any report on its expression and function at the beginning of identification. Next, through preparation of antibodies and recombinant proteins, Subfatin protein was detected in both culture medium of Subfatin-overexpressed cells and incubation medium of adipose tissue, identifying Subfatin is really a secreted protein and certainly a novel adipokine. Further, the tissue expression patterns of Subfatin displayed the highest in subcutaneous white adipose tissue among the detected 15 tissues from mouse and human, and scarce in the mouse brain, which was contrary to the tissue expression patterns of Meteorin, a homologue of Subfatin. No difference was observed in Subfatin expression level between adipocytes and stromal cells. Within adipose, Subfatin was located diffusely throughout the adipose tissue except lipid droplets. Under certain conditions, Subfatin expression was changeable, downregulated in white adipose tissue of CR rats, and dramatically upregulated during white adipocyte differentiation as well as in white adipose tissue of obese mice.

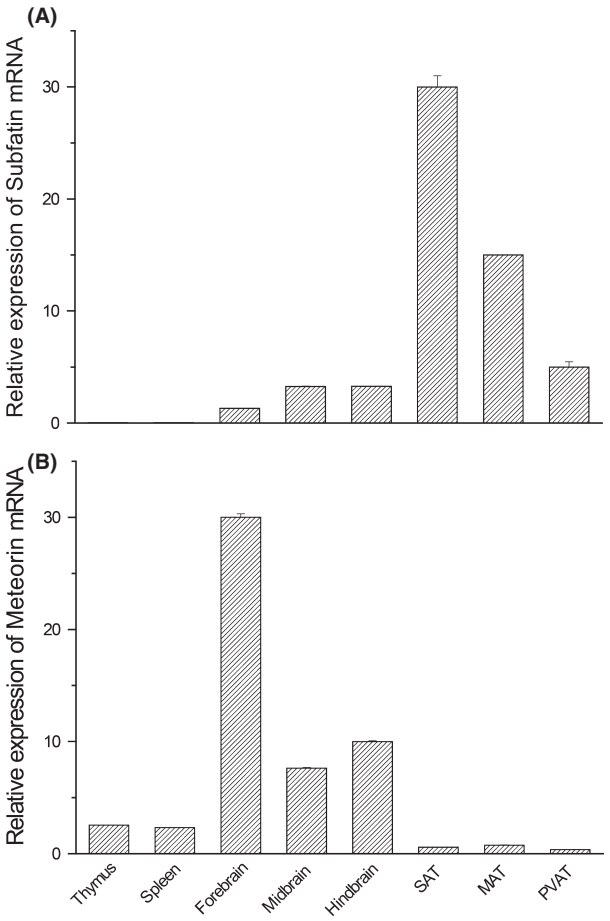

Figure 6 Different tissue expression patterns of Subfatin (**A**) and Meteorin (**B**) in mice (n = 3).

Adipokines have been shown to play crucial roles in the regulation of metabolism and in the pathogenesis of cardio-celebrovascular disease [3,12]. We speculate that adipokines participate in the beneficial effects of CR. Among the list of CR-regulated putative adipokines, Subfatin interests us for its high expression in adipose tissue, conservation during evolution, and homology to Meteorin that regulates glial cell differentiation [23]. Another very important reason for our interest is that there was no report on Subfatin expression or function at the beginning of identification, which gave us an opportunity as well as a challenge to verify it as a totally novel adipokine.

Subfatin molecular weight varies in the present study, which could be caused by mRNA splicing or posttranslational modifications, such as glycosylation and proteolysis. According to gene information analysis, there is a speculated splicing isoform of Subfatin, which lacks aa 1–82 and the signal peptide, suggesting the splice variant could not be secreted from cells. The higher molecular mass of Subfatin in HEK-293 lysate compared with that in COS-7 lysate might be due to glycosylation, which could result in the increased amounts of secreted Subfatin from HEK-293 cells, as N-glycosylation in general facilitates protein secretion from the cells [29]. Apart from the band with the expected molecular mass, the other band with a molecular weight lower than nonglycosylated Subfatin is recognized by anti-Subfatin antibody in the incubation medium of adipose tissue, suggesting that endogenous Subfatin may be digested in tissue or other posttranslational modifications exist in recombinant protein. Further, a band at about 55 kDa could be detected by Subfatin antibody (data not shown), suggesting a dimerization of Subfatin. Monoclonal antibody with higher specificity and affinity is needed to characterize the Subfatin protein in further research.

We investigated expression patterns of Subfatin in various tissues. Subfatin was highly expressed in adipose tissue rather than brain. Further results showed that Subfatin was much more highly expressed in subcutaneous adipose tissue, compared with perivascular and interscapular adipose tissues. Perivascular and interscapular adipose tissues are mainly brown adipose tissue, while subcutaneous adipose tissue is white adipose tissue [3,8]. Thus, Subfatin expression is higher in white adipose tissue than in brown adipose tissue. White adipose tissue deposits excessive energy, while brown adipose tissue burns surplus energy; so we guess that this adipokine may be associated with energy storage. Consistent with the assumption, both adipogenesis and diet-induced obesity increase its expression. These results further suggest that Subfatin may be a novel adipokine involved in adipogenesis and obesity. As hypothalamus plays an important role in metabolic homeostasis and obesity, more precise determination of Subfatin expression in brain remains to obtain in the further works.

It was also noted Subfatin expression in MAT and OAT was lower than that in SAT, although they were all white adipose tissue. The different expression levels in different depots of white adipose tissue could be due to adipose tissue heterogeneity between subcutaneous and visceral adipose tissue. Alternatively, these differences may result from increased nonadipocyte cells in visceral adipose tissue, for example mesenteric adipose tissue contained mesenteric blood vessels. In addition, the concentration of Subfatin in the serum/plasma is still unclear due to the lack of reliable methods until now. It remains to be determined in the future work, which may be helpful for predicting if Subfatin functions locally or systemically.

Subfatin expression is downregulated after treatment with adipocyte differentiation medium containing IBMX, insulin, and dexamethasone, suggesting these factors with their downstream signal molecules cannot induce Subfatin expression in preadipocytes. This is validated by assessing Subfatin expression after treating preadipocyte with these factors separately in our work (data not shown). After preadipocyte differentiation, Subfatin expression dramatically increased. So we speculate that the expression of Subfatin in adipocytes might be higher than that in the stromal cells in white adipose tissue. However, no significant difference was observed in Subfatin expression between adipocytes and stromal cells. Macrophages exist in adipose tissue and play a pivotal role in the adipose inflammation. We compared the Subfatin expression between cell line macrophages and mature adipocytes differentiated from preadipocytes, finding that the Subfatin expression in macrophages was extremely lower. These results indicate other types of cells in adipose stromal cells also highly express Subfatin.

Subfatin and Meteorin constitute a conserved protein family. And no well-annotated domains or structures have been identified in these two proteins, suggesting they are a novel protein family. Several studies have shown that Meteorin is a new neurotrophic factor that is expressed in undifferentiated neural progenitors and the astrocytes. Meteorin promotes neurite outgrowth, glial cell differentiation [23], and neurogenesis [30] and attenuates angiogenesis through upregulation of thrombospondin-1/-2 in astrocytes [31]. To our knowledge, there was no research on Subfatin expression or function at the beginning of the present study. Even now, there have been very limited reports on Subfatin, which were published most recently and related to brain research [32,33]. In 2012, one study reports that Subfatin promotes neurite outgrowth and neuroblast migration [32]. This function seems similar to Meteorin. However, different from Meteorin, Subfatin is expressed in the restricted sites of the brain such as inner ear during development, but apparently not in the adult nervous system [32]. Importantly, that study demonstrates a neuroprotection of Subfatin against deafness in guinea pigs [32]. Meanwhile, another study reports a similar function that Subfatin is required for neurite extension in rat hippocampal neurons [33]. Nevertheless, the expression of Subfatin in different adipose tissues and whether Subfatin is an adipose-specific adipokine are still unclear. Therefore, it is of importance for our present study demonstrating Subfatin is a novel adipokine and unlike Meteorin in adipose and brain expression.

As adipokine plays a key role in metabolism, our further work will describe the importance of Subfatin in metabolic homeostasis using fat-specific knockout and overexpression mice that have been successfully created in our laboratory. The potential significance of this novel adipokine in metabolic and cardio-cerebro-vascular diseases remains to be explored. The functional essence of this novel adipokine merits intensive investigation. In particular, does it act as a ligand exerting function through receptor signaling pathway (e.g., leptin, adiponectin and resistin)? Or, does it act as an enzyme exerting function through enzymatic reaction pathway (e.g., visfatin, adipsin, and lipase)?

Acknowledgments

This work was supported by grants from the National Basic Research Program of China (2009CB521902 to C.-Y.M.) and the National Natural Science Foundation of China (81130061 to C.-Y.M., 81202572 to Z.-Y.L. and 81373414 to C.-Y.M.).

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Table S1. Primer sequences.
Figure S1. Image of prokaryotic expression vector pET-21a(+).
Figure S2. Subfatin was subcloned into eukaryotic expression vector pCI-neo.
Figure S3. CR regulates the expression of Subfatin in rat adipose tissue.
Figure S4. Identification of Subfatin mammalian recombinant proteins with mass spectrometry.
Figure S5. Blank control picture of immunohistochemistry assay in adipose tissue for detecting localization and distribution of Subfatin. Bar = 100 μm.