Fat/Vessel-derived Secretory Protein (Favine)/CCDC3 Is Involved in Lipid Accumulation*

Received for publication, June 27, 2014, and in revised form, January 19, 2015 Published, JBC Papers in Press, January 20, 2015, DOI 10.1074/jbc.M114.592493

Sachiko Kobayashi†, Atsunori Fukuhara‡,¹, Michio Otsuki†, Takayoshi Suganami§, Yoshihiro Ogawa¶, Eiichi Morii‡ and Ichiro Shimomura†

From the Departments of †Metabolic Medicine and ‡Pathology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka and the Departments of §Organ Network and Metabolism and ¶Molecular Endocrinology and Metabolism, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-545 Yushima, Bunkyo-ku, Tokyo, Japan

Background: Coiled-coil domain-containing 3 (Ccdc3) mRNA is highly expressed in adipose tissue and vessels, but its function remains unknown.

Results: CCDC3 overexpression promoted adipogenesis of 3T3-L1 adipocytes, whereas CCDC3 deficiency reduced adiposity and inhibited age-related hepatic steatosis in mice.

Conclusion: CCDC3 positively regulates lipid accumulation.

Significance: CCDC3 is a possible therapeutic target to age-related obesity and diseases.

We previously identified a novel gene encoding Favine/CCDC3 (NCBI protein entry NP_083080), a possible secretory factor, the mRNA of which is highly expressed in adipose tissue and the aorta. The Favine mRNA levels are increased in the course of differentiation of rat primary adipocytes and are more elevated in the adipose tissue of genetically obese and diet-induced obese mice than in lean mice. However, its biological function has not yet been elucidated until now. Here, we tested the hypothesis that Favine is involved in lipid metabolism in adipocytes. We found that overexpression of Favine promoted 3T3-L1 adipocyte differentiation. To further investigate the function of Favine in vivo, we generated Favine knock-out (KO) mice. Favine KO mice exhibited a lean phenotype as they aged. The weights of white adipose tissue and liver were less, and adipocyte size was smaller in Favine KO mice compared with wild-type littermates (WT). Expression levels of lipogenic genes, such as fatty-acid synthase (FAS), acetyl-CoA carboxylase (ACC1), and diacylglycerol O-acyltransferase-2 (Dgat2), were decreased in adipose tissue of Favine KO mice. In 1-year-old mice, Favine deficiency decreased the number of inflammatory cells in white adipose tissue and diminished hepatic steatosis. In vitro, deficiency of Favine attenuated differentiation of primary adipocytes. Taken together, these data demonstrate that Favine has adipogenic and lipogenic effects on adipocytes.

Adipose tissue serves as a reservoir for energy and is also considered as an endocrine organ (1). Adipocyte differentiation, also called adipogenesis, is a complex and highly orchestrated program, which is accompanied by morphological changes, expression of adipocyte-specific genes and many lipogenic genes, extensive lipid accumulation, and the establishment of sensitivity to many hormones (2–4). In differentiated adipocytes, excess carbohydrate is stored as fat via lipogenesis, which is the metabolic process in synthesis of fatty acids and triglyceride (5, 6). An increase in both adipogenesis and lipogenesis is an important contributor to increased fat mass (7). In diet-induced obesity, increased lipid burden in adipose tissue and ectopic lipid accumulation in non-adipose tissues causes multiple dysfunction of the organs (8). Recently, aging has also been recognized to be linked with obesity (9, 10).

Previously, we searched for novel secretory factors associated with obesity and identified coiled-coil domain-containing 3/fat/vessel-derived secretory protein (CCDC3/Favine). Favine is a secretory protein predominantly expressed in adipose tissue and the aorta. The expression levels of Favine mRNA are increased in murine adipose tissue of genetic and diet-induced obesity models and induced in the course of differentiation of rat primary adipocytes. In differentiated adipocytes, mRNA expression levels of Favine are augmented by insulin and peroxisome proliferator-activated receptor γ (PPARY) agonist and suppressed by TNF-α, norepinephrine, and isoproterenol (11). Eberlein et al. (12) showed that mRNA expression levels of Favine in bovine muscle are correlated with the amount of intramuscular fat deposition. Ugi et al. (13) observed that human omental Favine mRNA is correlated to body mass index and waist circumference. Although these observations suggest that Favine is involved in lipid accumulation, the function of Favine has not been elucidated.

Here, we show for the first time that Favine has adipogenic and lipogenic effects by gain-of-function study in murine 3T3-L1 preadipocyte cell line and loss-of-function study in mice.

* This work was supported by Grant-in-aid for JSPS Fellows 215490, Grant-in-aid for Young Scientists 25860767, and Grants-in-aid for Scientific Research 24390238 from the Japan Society for the Promotion of Science and a grant from Japan Foundation for Applied Enzymology and from the Japan Health Foundation.

† To whom correspondence should be addressed. Tel.: 81-6-879-3732; Fax: 81-6-879-3739; E-mail: fukuhara@endmet.med.osaka-u.ac.jp.

‡ The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; CAR, Coxsackie-adenovirus receptor; FAS, fatty-acid synthase; ACC1, acetyl-CoA carboxylase α; Dgat, diacylglycerol O-acyltransferase; ChREBP, carbohydrate-responsive element-binding protein; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; C/ebpα, CCAAT/enhancer-binding protein α; Pref-1, preadipocyte factor-1; Ctgf, connective tissue growth factor; Favine, fat/vessel-derived secretory protein.

1 To whom correspondence should be addressed. Tel.: 81-6-879-3732; Fax: 81-6-879-3739; E-mail: fukuhara@endmet.med.osaka-u.ac.jp.
Favine and Lipid Accumulation

EXPERIMENTAL PROCEDURES

Animals—Mice had ad libitum access to water and chow (MF, Oriental Yeast, Saitama, Japan). All animals were housed in a temperature-controlled room under a 12-h light/12-h dark cycle. Animals were weighed at 10 a.m. in the fed state. Food intake was determined in individually housed mice by weighing remaining diet. Mice were sacrificed at 1 p.m. Tissues of each mouse were dissected out, washed with phosphate-buffered saline (PBS), and immediately weighed and deposited into liquid nitrogen or into 4% buffered formaldehyde. Male Favine KO mice and wild-type littermates were used for experiments. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Cell Cultures—3T3-L1 cells were purchased from National institute of Biomedical Innovation, JCRB Cell Bank (Japan), and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Two days post-confluence, the cells were treated with adipogenic mixture containing 0.5 mM 3-isobutyl-1-methylxanthine (Nacalai Tesque, Kyoto, Japan), 1 μM dexamethasone (Sigma), and 0.1 or 1 μM insulin (Nacalai Tesque) for 48 h. After 48 h, medium was replaced with DMEM supplemented with 10% FBS. The stable Coxsackie-adenovirus receptor (CAR)-expressing 3T3-L1 adipocytes (CAR-3T3-L1 adipocytes) were maintained in DMEM supplemented with 10% FBS and 2 μg/ml puromycin (Nacalai Tesque) and differentiated into mature adipocytes with adipogenic mixture containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μM insulin for 48 h (14). HEK293A cells and HEK293T cells were maintained in DMEM supplemented with 10% FBS.

Generation of Stable Favine-overexpressing 3T3-L1 Cells—The retroviral vector, pMXs-IG, and Plat-E cells were kindly provided by Dr. T. Kitamura (Institute of Medical Science, the University of Tokyo) (15). C-terminal His-tagged Favine was inserted into pMXs-IG (named as pMXs-IG-Favine). The retroviral vectors, empty pMXs-IG and pMXs-IG-Favine, were transduced into Plat-E cells using Lipofectamine 2000 reagent (Invitrogen). 48 h after transfection, the supernatants were harvested as viral stock solutions. 3T3-L1 cells stably expressing Favine were obtained by infection with viral stocks, followed by a collection of GFP-positive cells with FACS Aria (BD Biosciences).

Generation of Transient Favine-overexpressing CAR-3T3-L1 Adipocytes—The adenoviral vector, pAD/CMV/V5-DEST Gateway vector, was purchased from Invitrogen. β-Galactosidase (LacZ) and Favine were inserted into the pAD/CMV/V5-DEST Gateway vector, respectively (named as pAD-LacZ and pAD-Favine). The adenoviral vectors, pAD-LacZ and pAD-Favine, were transfected into HEK293A cell lines to produce adenoviral stock (named as Ad-LacZ and Ad-Favine, respectively). Amplified adenoviral stock was purified by Adeno-X Maxi purification kit (Clontech) as per the manufacturer’s instructions. The CAR-3T3-L1 adipocytes on the 4th day after induction were transfected with Ad-LacZ and Ad-Favine, incubated for 3 days, and used for lipogenesis assay and oxygen consumption assay.

Lipogenesis Assay—The stable Favine-overexpressing 3T3-L1 adipocytes and adenovirus-infected CAR-3T3-L1 adipocytes were serum- and glucose-deprived and assayed as described (16) with some modifications. Cells were incubated for 3 h prior to assay in Krebs-Ringer bicarbonate buffer supplemented with 30 mM HEPES, 0.5% bovine serum albumin (KRBH/BSA), and 2.5 mM glucose. The cells were washed once with PBS and incubated for another 15 min in KRBH/BSA without glucose. 100 nM insulin were added, and the cells were incubated for 15 min. The reaction was initiated by the addition of [U-14C]glucose (1 μCi/well) and glucose (5 mM final concentration). The assay was terminated after 1 h by washing three times with ice-cold PBS. Radiolabeled glucose incorporation into lipid was assayed by placing cells into 0.5 ml of PBS and shaking vigorously with 2.5 ml of Betalfluor Liquid Scintillation Fluid (National Diagnostics). After samples settled overnight, radioactivity in 1.5 ml of the organic phase was determined by scintillation counting.

Western Blot Analysis—Cell lysates and culture media of 3T3-L1 cells were harvested. Culture media were concentrated by absorption onto a nickel resin (GE Healthcare). Nickel-Sepharose-bound proteins and 10 μg of cell lysates were subjected to SDS-PAGE under reducing conditions, transferred, and blotted with HRP-conjugated anti-His antibody (Qiagen, Santa Clarita, CA) or anti-rabbit GFP antibody 2956 (Cell Signaling Technologies, Danvers, MA) followed by HRP-conjugated rabbit IgG antibody (Amersham Biosciences). The membrane was stripped and reprobed with anti-mouse β-actin Clone AC-15 (Sigma), followed by HRP-conjugated mouse IgG antibody (Amersham Biosciences).

Oil Red O Stains in Adipocytes—Lipid accumulation in adipocytes was examined by Oil Red O staining (17). Cells were fixed with 4% formaldehyde, washed with water, dried, and stained with 0.18% Oil Red O (Nacalai Tesque) in 60% isopropl alcohol (Nacalai Tesque). Stained cells were washed with water and dried. The tissue culture plates were scanned on a flatbed scanner. Dye was extracted using dimethyl sulfoxide (Nacalai Tesque), and its absorbance was determined at 540 nm.

RNA Isolations—Total RNA was extracted from each tissue using RNA STAT-60 (Tel-Test, Friendswood, TX) or Sepasol®-RNA I Super G (Nacalai Tesque) according to the instructions supplied by the manufacturers.

Quantitative Real Time RT-PCR—Quantitative real-time RT-PCR was performed as described previously (11). The sequences of primers used in real time RT-PCR are listed in Table 1.

Targeted Disruption of Ccdc3/Favine Gene—Favine KO mice were generated at Unitech, Co. Ltd. (Chiba, Japan). The Ccdc3/Favine chromosomal gene was isolated from bacterial artificial chromosome clones of C57BL/6J mice, and its exon1 and most of exon2 were replaced by a coding neomycin resistance (Neo) gene (shown in Fig. 2A). Embryonic stem cells were electroporated, and 7 of 41 screened colonies were found to be correctly targeted, followed by injection into C57BL/6J blastocysts to generate chimeric mice. The chimeric mice were crossed with C57BL/6J mice to obtain heterozygous Favine KO mice. Mice were genotyped by PCR using two Favine-specific primers (primer A, 5’-AGG TCT GGT GTT CAA GGC GTT G-3’;
Favine and Lipid Accumulation


table 1  
sequences of PCR primers used for quantitative real time RT-PCR

| Primer | Forward | Reverse |
|--------|---------|---------|
| 36B4   | 5'-GCCTCACGAGTGAAGCTTGA-3' | 5'-GCCTCTTGAAGTGAAGCTTGA-3' |
| CypA   | 5'-CAGAAGAAGTGAAGCTTGA-3' | 5'-CAGAAGAAGTGAAGCTTGA-3' |
| β-actin| 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| Favine | 5'-GGATAGTGAAGCTTGA-3' | 5'-GGATAGTGAAGCTTGA-3' |
| FA5    | 5'-CAGAAGAAGTGAAGCTTGA-3' | 5'-CAGAAGAAGTGAAGCTTGA-3' |
| Dgat1  | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| Dgat2  | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| ChREBP | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| ChREBP-β| 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| SREBP-1c| 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| MTP    | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| Cidea2 | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| ACC1   | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| PPARy  | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| adiponectin| 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| Ap2    | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| Pref-1 | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| TGF-β1 | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |
| Ctgf   | 5'-GCTATGAGTGAAGCTTGA-3' | 5'-GCTATGAGTGAAGCTTGA-3' |

primer B, 5'-TGG GTG CAC GGC TAG CTC TTC G-3') and a Neo-specific primer (primer C, 5'-ACC GAC CAA GCG ACG TCT GA-3') shown in Fig. 2A. For Southern blot, genomic DNA was isolated, digested with SacI or AseI, and detected using two probes in Fig. 2A.

Measurements of Parameters in Murine Plasma—Blood samples were collected from tail veins. Plasma glucose, triglyceride, insulin, free fatty acid, and leptin concentrations were measured using the glucose CII-test kit, triglyceride E-test (Wako), and mouse leptin ELISA kit (Morinaga), respectively, according to the manufacturers' instructions. Other plasma parameters were measured using Vetscan VS2 (Abaxis, Union City, CA).

Insulin tolerance Test—After 4 h of fasting, mice were injected with 1.0 milliunits/g body weight of human regular insulin intraperitoneally. Blood samples were collected from tail veins every 15–30 min for 120 min, and blood glucose was measured.

Measurements of Triglyceride in Murine Livers—Hepatic triglyceride was extracted as described (18) with some modifications. Adipose tissue was minced and digested at 37 °C in DMEM containing 20 mg of collagenase (Sigma). After digestion, the tissue remnants were removed by filtration through a 250-μm filter. Cells were suspended with DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 mM pioglitazone (Takeda), and 200 μM-l-ascorbic acid and then filtered through a 25-μm filter. Isolated cells were plated on a collagen type 1 plate. Medium was replaced every 2 days. Two days after 100% confluency, the cells were treated with adipogenic mixture containing 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μM dexamethasone, 1 μM insulin, and 5 μM pioglitazone (Takeda Pharma). After 48 h, medium was replaced with DMEM containing 0.1 μM insulin and 10% FBS.

Measurement of Adipocyte Cell Size—Adipocyte cell size was measured using BZ-X700 (KEYENCE, Osaka, Japan). The adipocyte area was measured using the Student's t test. A p value less than 0.05 was considered statistically significant.

RESULTS

Retroviruses-mediated Overexpression of Favine Enhanced Adipogenesis and Lipogenesis in 3T3-L1 Cells—To elucidate the functions of Favine in adipocytes, we generated 3T3-L1 cells stably expressing Favine. 3T3-L1 cells were transduced with pMXs-Favine-IREs-GFP retrovirus (Favine-L1 cells) or pMXs-IREs-GFP retrovirus (Mock-L1 cells). Approximately equal amounts of intracellular GFP expression were confirmed between Favine-L1 cells and Mock-L1 cells by Western blot analysis (Fig. 1A, upper panel). Favine was detected in intracellular protein, and Favine-L1 cells secreted Favine into culture media as a 37-kDa protein (Fig. 1B, upper panel). β-Actin was blotted as loading controls for intracellular proteins (Fig. 1A, lower panel, and B, lower panel). Morphologic differences were not observed between undifferentiated Favine-L1 cells and Mock-L1 cells, and the proliferation rate was similar between two types of cells (data not shown). Interestingly, after adipogenic induction, Oil Red O staining showed that the number of lipid-laden cells was increased in Favine-L1 cells compared with Mock-L1 cells (Fig. 1C). The size of the lipid drop was similar between Mock-L1 cells and Favine-L1 cells (Fig. 1C, upper panel). Then we measured the expression of genes involved in adipocyte differentiation and lipid metabolism. The mRNA expression levels of PPARγ, which is the master regulator of adipogenesis, adi-
Favine and Lipid Accumulation

**FIGURE 1.** Favine overexpression in 3T3-L1 cells. A, Western blot of GFP (upper panel) and β-actin (lower panel) in cell lysates from undifferentiated Favine-L1 cells and Mock-L1 cells. B, Western blot of His-tagged Favine (upper left panel) and β-actin (lower left panel) in cell lysates and His-tagged Favine in culture media (right panel) from undifferentiated Favine-L1 cells and Mock-L1 cells. C, Oil Red-O stains of Favine-overexpressing 3T3-L1 cells. Mock-L1 cells and Favine-L1 cells were treated with adipogenic mixtures and stained with Oil Red-O on the 6th day after induction (upper panel, original magnification, ×200; lower left panel, scanning of stained culture plate). Extraction of the absorbed dye using DMSO and absorbance measurements at 540 nm (lower right panel). Data are mean ± S.D.; ***p < 0.001 versus Mock-L1 cells. D, lipogenic activity in Favine-L1 cells. OD540. (**C)Glucose incorporation into lipid on the 7th day after induction was determined (n = 6). Data are mean ± S.D.; ***p < 0.001 versus Mock-L1 cells.

In Adipose Tissue of Favine KO Mice, de Novo Lipogenesis Was Down-regulated and Age-related Adiposity Was Attenuated with Less Inflammatory Cells—For the study on leanness of Favine KO mice, mice were prepared for histological analysis at 14 weeks of age (young stage) and at 1 year of age (aged stage), respectively. At 14 weeks of age, the weight of epididymal and subcutaneous adipose tissue was significantly less in Favine KO mice compared with WT mice (Fig. 3A). Adipocyte size in both epididymal and subcutaneous adipose tissue was smaller in KO mice (Fig. 3B, upper panel, and C), which could account for the decrease of the fat mass. At 1 year of age, in epididymal adipose tissue of Favine KO mice the number of inflammatory cells was decreased as well compared with WT mice (Fig. 3, lower panel).

Results of the glucose tolerance test and the insulin tolerance test in offspring was confirmed by both Southern blot and PCR analysis (Fig. 2D). The absence of Favine mRNA in the knock-out mouse was validated by quantitative real time RT-PCR with cDNA generated from epididymal adipose tissue, aorta, liver and muscle RNA from F3 animals (Fig. 2C).

Favine KO Mice Showed Lean Phenotype—Favine KO mice were viable, fertile, and showed normal development. The body weight of Favine KO mice was the same as that of controls at 6 weeks of age, suggesting no congenital growth retardation. There was a trend toward a decrease of body weight as they got aged under a normal chow diet, and in the later stage Favine KO mice showed a statistically significant lean phenotype (Fig. 2D). Analysis of food intake did not reveal any differences between WT and Favine KO mice (Fig. 2E). Plasma glucose levels in the fed state were slightly higher in Favine KO mice compared with WT mice (Fig. 2F). Plasma insulin (data not shown), triglyceride, and free fatty acid levels of KO mice did not differ (Table 3). Results of the glucose tolerance test and the insulin tolerance test showed no significant difference at the 16 weeks of age between the two genotypes (data not shown), whereas 9-month-old KO mice showed higher insulin sensitivity (Fig. 2G).
significantly reduced compared with WT mice, besides a reduced fat mass with smaller adipocytes (Fig. 3B, lower panel).

We next measured the expression of genes involved in adipocyte differentiation and lipid metabolism in epididymal adipose tissue. The expression of genes involved in adipocyte differentiation, such as PPARγ and adiponectin, was not affected (Table 2, middle), suggesting that the decreased adipocyte size was not due to reduced adipocyte differentiation. The levels of plasma adiponectin in Favine KO mice were not changed, but the levels of plasma leptin in Favine KO mice were decreased (Table 3). Interestingly, expression levels of lipogenic genes (FAS, ACC1, and Dgat2) were significantly attenuated in epididymal adipose tissue of KO mice compared with WT mice (Table 2, middle), although mRNA levels of lipolytic genes (adipose triglyceride lipase (Atgl) and hormone-sensitive lipase (HSL)) were not altered (data not shown). Actually, basal lipolysis, fasting-induced lipolysis, and isoproterenol-stimulated lipolysis were unchanged in Favine KO mice (data not shown). ChREBP-β, a recently identified isoform of ChREBP in adipose tissue (20), was extremely down-regulated in KO mice. Total ChREBP expression, the sum of canonical isoform α and newly identified isoform β, in KO mice tended to be declined. SREBP-1c mRNA remained unchanged (Table 2, middle).

**Lipid Accumulation in Livers of Aged Favine KO Mice Was Diminished**—Liver is recognized to be a major lipogenic organ. Favine KO mice showed a lean phenotype (Fig. 2D) with reduced hepatic weight (Fig. 3A). Then, hepatic phenotypes were evaluated.

At 14 weeks of age, hepatic histological appearance was not different between genotypes (data not shown), and triglyceride contents in livers were not changed (Fig. 4A). Expression levels of three lipogenic genes in the liver; FAS, Dgat2, and microsomal triglyceride transfer protein (MTP), were not altered in Favine KO mice compared with WT mice (Fig. 4B).

---

**TABLE 2**

Summary of quantitative real time RT-PCR

| Overexpression in 3T3-L1 adipocytes | Adipose tissue in mice | Primary adipocytes from mice |
|------------------------------------|-----------------------|-----------------------------|
| **Mock-L1**                        | **Favine-L1**         |                             |
| PPARγ                              | 1.0±0.02              | 1.61±0.17                  |
| adiponectin                        | 1.0±0.05              | 1.61±0.11                  |
| C/ebpA                             | 1.0±0.02              | 1.46±0.27                  |
| Ap2                                | 1.0±0.10              | 2.54±0.60                  |
| **FAS**                            | 1.0±0.08              | 1.15±0.04                  |
| **ACC1**                           | 1.0±0.14              | 1.28±0.13                  |
| **Dgat1**                          | 1.0±0.10              | 1.75±0.02                  |
| **Dgat2**                          | 1.0±0.07              | 1.98±0.04                  |
| **Lipogenic Transcription Factors**|                       |                             |
| ChREBP total                       | 1.0±0.12              | 0.95±0.05                  |
| ChREBP-β                           | n.d.                  | n.d.                       |
| SREBP-1c                           | 1.0±0.30              | 1.4±0.06                   |

Data are mean ± S.D. or ± S.E. n.d. indicates not detected. +, p = 0.07; #, p = 0.06; *, p < 0.05; †, p < 0.01; ‡, p < 0.001 versus Mock or WT.
At 1 year of age, we found that hepatic steatosis was markedly diminished in Favine KO mice, although livers of WT mice had increased the number of lipid droplets in hepatocytes and developed age-related steatosis (Fig. 4, C and D). Actually, amounts of hepatic triglycerides of aged KO mice were significantly less compared with aged WT mice (Fig. 4A). It is of inter-
Favine and Lipid Accumulation

Table 4

Quantitative real time RT-PCR in primary undifferentiated adipocytes

|     | WT       | KO        |
|-----|----------|-----------|
| Pref-1 | 1.0±0.10 | 0.93±0.13 |
| TGF-β1 | 1.0±0.054 | 1.45±0.071* |
| Ctgf  | 1.0±0.072 | 2.6±0.069* |

**Discussion**

In this study, overexpression of Favine in 3T3-L1 cells enhanced adipocyte differentiation with increased expression of the master regulator of adipogenesis, PPARγ, and the adipocyte differentiation markers, adiponectin and Ap2. On the contrary, deficiency of Favine in primary adipocyte culture caused impairment of adipocyte differentiation with low expressions of PPARγ, C/ebpα, adiponectin, and Ap2. These data suggest that Favine is an adipogenic factor in vitro. We also found that mRNA levels of two anti-adipogenic factors, TGF-β1 and Ctgf, were increased in Favine-deficient primary preadipocytes (21, 22, 24). TGF-β inhibits differentiation of 3T3-L1 cells into mature adipocytes at the early stage (25) and inhibits lipid accumulation in porcine primary adipocytes (26). CTGF is a downstream mediator of TGF-β signaling and has anti-adipogenic effects in murine primary adipocytes (22).

There is tight inter-regulation of adipogenic transcription and lipogenesis (4). The expression levels of a series of lipogenic enzymes were enhanced in differentiated stable Favine-overexpressing 3T3-L1 cells and attenuated in differentiated Favine-deficient primary adipocytes, indicating that Favine is involved in lipogenesis in vitro. Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in preadipocytes of 3T3-L1 cells was impaired—Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of PPARγ, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis in vitro. Although we observed that Favine overexpression in pread-
Favine KO mice exhibited a lean phenotype with reduced fat mass and smaller adipocyte size compared with WT mice. In adipose tissue of Favine KO mice, expression levels of lipogenic genes (FAS, ACC1, and Dgat2) were profoundly down-regulated, although expression level of PPARγ, which is essential not only for adipogenesis but also post-differentiation and survival of mature adipocytes in vivo (27, 28), was intact, and adiponectin mRNA and plasma adiponectin levels were not affected. We infer that the decrease of leptin was due to reduced fat mass. Reduced lipogenesis without impairment of adipocyte differentiation implies that Favine may be preferentially involved in lipogenesis in vivo. We speculate that some factors might compensate for loss of Favine-mediated adipogenic effects in vivo. In our preliminary experiments, hepatic overexpression of Favine induced the expressions of lipogenic genes such as FAS and Dgat2 without significant increase of PPARγ mRNA, indicating that Favine could also have lipogenic effects in non-adipose tissues in vivo (data not shown).

ChREBP has been recognized as a central regulator of glycolysis and de novo lipogenesis in liver (29). Newly identified ChREBP-β is a highly active isoform of ChREBP in adipose tissue (20). ChREBP-β, induced by glucose or glucose metabolite, regulates the expression levels of lipogenic enzymes such as FAS and ACC1 in adipose tissue. ChREBP null KO mice show modest glucose intolerance with hyperglycemia, accompanied by reduced adipose tissue weight (30). Our data showed that ChREBP-β expression was markedly reduced in adipose tissue of Favine KO mice, suggesting that Favine deficiency reduces transcription of ChREBP-β followed by coordinated down-regulation of de novo lipogenic genes. Favine KO mice developed a mild hyperglycemia in the fed state for reasons as yet unexplained. Down-regulation of adipose ChREBP may partially explain metabolic changes of both de novo lipogenesis in adipose tissue and glucose homeostasis in Favine KO mice.

Besides reduced lipogenesis per se in adipose tissue, changes in energy output and catabolic property of adipose tissue in Favine KO mice were possible mechanisms for reduced body weight and adipose tissue mass. In this study, basal oxygen consumption rate in Favine-overexpressing CAR-3T3-L1 differentiated adipocytes and lipolysis in Favine KO mice were unchanged (data not shown). Further analysis on metabolic states in other tissues will clarify the leanness of Favine KO mice.

Advancing age is accompanied by excessive lipid accumulation in not only adipose tissue but also non-adipose tissue such as the liver. Aging is also recognized to be linked with chronic inflammatory conditions in adipose tissue, resulting in insulin resistance (9, 10). However, the underlying mechanisms of age-related development of ectopic lipid accumulation and adipose tissue inflammation have been poorly understood. In this study, Favine deficiency in mice strongly inhibited age-related hepatic steatosis and decreased the number of inflammatory cells in epididymal adipose tissue, which could partly explain higher insulin sensitivity in aged Favine KO mice compared with WT mice, whereas the young KO mice showed normal insulin sensitivity.

The expression of hepatic mRNA involved in lipid metabolism did not change in either 14-week-old or 1-year-old Favine KO mice. However, the expressions of lipogenic genes in adipose tissue were decreased, and adipose tissue weight was reduced in Favine KO mice compared with WT mice. These data indicate that adipose tissue should be involved in protection of hepatic steatosis in Favine KO mice. One possible mechanism is that lack of Favine secretion from adipose tissue might inhibit hepatic steatosis in an endocrine manner. The other is that reduced fatty acid entry into liver repressed hepatic lipid accumulation in Favine KO mice.

We previously reported that Favine is expressed highly in murine aorta (11). In this study, Favine KO mice showed no morphological changes in the aorta, and blood pressure in Favine KO mice was similar to that of WT mice. The number of vessels stained with CD31 (31), one of endothelial cell markers, in adipose tissue in Favine KO mice was not changed (data not shown). Recently Azad et al. (32) showed that Favine represses tumor necrosis factor-α-stimulated vascular cell adhesion molecule-1 expression in endothelial cells in vitro. Further investigation on the function of Favine in endothelial cells in vivo and in vitro will help with understanding the mechanism of physiological action of Favine. To establish the system to measure plasma Favine levels will provide valuable information for significance of this factor in obesity and age-related diseases, which is now underway.

In summary, Favine is involved in adipogenesis and lipogenesis in adipose cells, and it also relates to age-related hepatic steatosis.

Acknowledgments—We thank Takako Sawamura for technical assistance. We also thank Dr. Yasuko Nishizawa for helpful advice, Dr. Yoshihiro Kamada for evaluation of murine liver, and Dr. Shun bun Kita for comments on the manuscript.

Note Added in Proof—Tables 1–4 were misnumbered in the version of this article that was published as a Paper in Press on January 20, 2015. This has now been corrected.

REFERENCES
1. Kershaw, E. E., and Flier, J. S. (2004) Adipose tissue as an endocrine organ. J. Clin. Endocrinol. Metab. 89, 2548–2556
2. Rosen, E. D., and Spiegelman, B. M. (2000) Molecular regulation of adipogenesis. Annu. Rev. Cell Dev. Biol. 16, 145–171
3. Lefterova, M. I., and Lazar, M. A. (2009) New developments in adipogenesis. Trends Endocrinol. Metab. 20, 107–114
4. Lowe, C. E., O’Rahilly, S., and Rochford, J. J. (2011) Adipogenesis at a glance. J. Cell Sci. 124, 2681–2686
Favine and Lipid Accumulation

5. Sethi, J. K., and Vidal-Puig, A. J. (2007) Thematic review series: adipocyte biology-adipose tissue function and plasticity orchestrate nutritional adaptation. J. Lipid Res. 48, 1253–1262
6. Kersten, S. (2001) Mechanisms of nutritional and hormonal regulation of lipogenesis. EMBO Rep. 2, 282–286
7. Strable, M. S., and Ntambi, J. M. (2010) Genetic control of de novo lipogenesis: role in diet-induced obesity. Crit. Rev. Biochem. Mol. Biol. 45, 199–214
8. Boreń, J., Taskinen, M. R., Olofsson, S. O., and Levin, M. (2013) Ectopic lipid storage and insulin resistance: a harmful relationship. J. Intern. Med. 274, 25–40
9. Tchkonia, T., Morbeck, D. E., Von Zglinicki, T., Van Deursen, J., and Lustgarten, J. S. (2010) Quantitation of adipose conversion and triglycerides by staining orglycogen synthesis in 3T3-L1 adipocytes. J. Biol. Chem. 285, 497–509
10. Ignotz, R. A., and Massague, J. (1985) Type II transforming growth factor (TGF-β) and adipsogenesis in pigs. J. Anim. Sci. 67, 2171–2180
11. Lee, K., Villena, J. A., Moon, Y. S., Kim, K. H., Lee, S., Kang, C., and Sul, H. S. (2003) Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor-1 (Pref-1). J. Clin. Invest. 111, 453–461
12. Tchkonia, T., Morbeck, D. E., Von Zglinicki, T., Van Deursen, J., Lustgarten, J., and Kumagai, H. (2003) Retrovirus-mediated gene transfer and expression of CCDC3/Favine in adipocytes and endothelial cells. Biochem. Biophys. Res. Commun. 392, 29–35
13. Valverde, J. L., Castro-Mun˜ozledo, F., and Kuri-Harcuch, W. (2010) Genetic control of de novo lipogenesis: role in diet-induced obesity. Crit. Rev. Biochem. Mol. Biol. 45, 199–214
14. Ito, A., Suganami, T., Miyamoto, A., Chen, M., and Ogawa, Y. (2007) Role of MAPK phosphatase-1 in the induction of monocyte chemoattractant protein-1 during the course of adipocyte hypertrophy. J. Biol. Chem. 282, 25445–25452
15. Kitamura, T., Koshino, Y., Shibata, F., Oki, T., Nakajima, H., Nosaka, T., and Kumagai, H. (2003) Retrovirus-mediated gene transfer and expression of CCDC3/Favine and Lipid Accumulation.
16. Eberlein, A., Kalbe, C., Goldammer, T., Brunner, R. M., Kuehn, C., and Weikard, R. (2010) Analysis of structure and gene expression of bovine CCDC3. Biochem. Biophys. Res. Commun. 392, 29–35
17. Wiese, R. J., Mastick, C. C., Lazar, D. F., and Saltiel, A. R. (1995) Activation of mitogen-activated protein kinase and phosphatidylinositol 3’-kinase is not sufficient for the hormonal stimulation of glucose uptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes. J. Biol. Chem. 270, 3442–3446
18. Ramírez-Zacarias, J. L., Castro-Muñozledo, F., and Kuri-Harcuch, W. (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic liquids with Oil red O. Histochemistry 97, 493–497
19. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509
20. Herman, M. A., Peroni, O. D., Villoria, J., Schön, M. R., Abumrad, N. A., Blüher, M., Klein, S., and Kahn, B. B. (2012) A novel ChREBP isoform in adipose tissue regulates systemic glucose metabolism. Nature 484, 333–338
21. Zamani, N., and Brown, C. W. (2011) Emerging roles for the transforming growth factor-β superfamily in regulating adiposity and energy expenditure. Endocr. Rev. 32, 387–403
22. Imai, T., Takakuwa, R., Marchand, S., Dentz, E., Bornert, J. M., Messaddeq, N., Wendling, O., Mark, M., Desvergne, B., Wahli, W., Chambon, P., and Metzger, D. (2004) Peroxisome proliferator-activated receptor γ knockout causes insulin resistance in fat and liver but not in muscle. Proc. Natl. Acad. Sci. U.S.A. 101, 15712–15717
23. Imai, T., Takakuwa, R., Marchand, S., Dentz, E., Bornert, J. M., Messaddeq, N., Wendling, O., Mark, M., Desvergne, B., Wahli, W., Chambon, P., and Metzger, D. (2004) Peroxisome proliferator-activated receptor γ is required in mature white and brown adipocytes for their survival in the mouse. Proc. Natl. Acad. Sci. U.S.A. 101, 4543–4547
24. Filhoulaud, G., Guilmeau, S., Dentin, R., Girard, J., and Postic, C. (2013) Novel insights into ChREBP regulation and function. Trends Endocrinol. Metab. 24, 257–268
25. Izuoka, K., Bruck, R. K., Liang, G., Horton, J. D., and Uyeda, K. (2004) Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. Proc. Natl. Acad. Sci. U.S.A. 101, 7281–7286
26. Newman, P. J., Berndt, M. C., Gorski, J., White, G. C., 2nd, Lydick, C., and Paddock, C., and Muller, W. A. (1990) PECAM-1 (CD31) clustering and relation to adhesion molecules of the immunoglobulin gene superfamily. Science 247, 1219–1222
27. Azad, A. K., Chakrabarti, S., Xu, Z., Davidse, S. T., and Fu, Y. (2014) Coiled-coil domain containing 3 (CCDC3) represses tumor necrosis factor-α/nuclear factor-κB-induced endothelial inflammation. Cell. Signal. 26, 2793–2800