The novel gene *fad*104, containing a fibronectin type III domain, has a significant role in adipogenesis

Kei Tominaga[a,b], Chiharu Kondo[a], Yoshikazu Johmura[a], Makoto Nishizuka[a], Masayoshi Imagawa[a,*]

[a] *Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan*

[b] *Research Division, Nissui Pharmaceutical Co., Ltd., 1075-2 Hokunanmoro, Yuki, Ibaraki 307-0036, Japan*

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Abstract A novel gene named *fad*104 (factor for adipocyte differentiation-104), whose expression level quickly increased in the early stage of adipogenesis, was isolated and characterized. The deduced amino acid sequence of *fad*104 revealed the possible presence of a fibronectin type III domain and transmembrane domain. The expression of *fad*104 was detected in adipocyte differentiable 3T3-L1 cells but not observed in the non-adipogenic cell line NIH-3T3. Moreover, the ability of 3T3-L1 cells to differentiate declined with the knockdown of *fad*104 by RNA interference, strongly indicating that *fad*104 functions as a positive regulator of adipogenesis.

Keywords: Adipocyte differentiation; Adipogenesis; Obesity; Peroxisome proliferator-activated receptor γ; Fibronectin type III domain; 3T3-L1 cell

1. Introduction

The key role of white adipose tissue is the storage of energy in the form of triglycerides. Adipose tissue provides adipocytokines for maintenance of the balance between energy intake and energy output [1]. Since the functions of these adipocytokines are disrupted in obese individuals, obesity is associated with a number of important diseases [2]. Adipocyte growth occurs with an increase in the size and number of adipocytes derived from the differentiation of precursor cells. Several lines of evidence revealed that peroxisome proliferator-activated receptor γ (PPARγ), some of the CCAAT/enhancer-binding protein (C/EBP) family and sterol regulatory element binding protein-1 (SREBP-1) function as master regulators of adipocyte differentiation.

The ectopic expression of PPARγ directs cells to become adipogenic [3]. C/EBPα was also identified as a master regulator in an ectopic expression experiment [4]. The positive feedback activity of C/EBPα against PPARγ is also important for the maintenance of the adipogenic state [5]. C/EBPβ and C/EBPδ are known to induce the expression of C/EBPα and PPARγ [6]. C/EBPβ was shown to participate in mitotic clonal expansion by loss of function experiments [7]. Until now, adipogenesis has been explained by a complex series of transactivations of target genes by these master regulators. However, it is impossible to elucidate the mechanism of adipogenesis based only on these factors. Moreover, their expression is observed from the mid phase to log phase of adipogenesis and the factors initiating differentiation at the earliest phase are poorly isolated and characterized.

Adipocyte differentiation is imitated by 3T3-L1 preadipocytes in vitro, in response to adipogenic inducers. In our laboratory, using this cell line, factors which were expressed 3 h after the stimulation were isolated. Of the 102 genes isolated [8,9], there included regulator of G protein signaling 2 (RGS2) [10], TC10-like/TC10βLong (TCL/TC10βL) [11], BACH1, ARA70 [9] and p68 RNA helicase [12]. Further investigation was done with RGS2, TCL/TC10βL and p68 RNA helicase, which have now been characterized as factors accelerating adipocyte differentiation [10–12].

In this study, we report the cloning and functional analysis of the gene *fad*104 (factor for adipocyte differentiation-104). *Fad*104 contains a fibronectin type III domain and transmembrane domain. Its expression was elevated during the adipogenesis of mouse 3T3-L1 cells. The expression of *fad*104 was strongly restricted to the adipocyte differentiable 3T3-L1 cells and was not detected in the non-adipogenic cell line, NIH-3T3. Moreover, when *fad*104 expression was knocked down using the RNA interference (RNAi) method, the ability of 3T3-L1 cells to differentiate declined. Taken together, these results strongly indicate that *fad*104 is another factor which possesses a significant role in adipocyte differentiation.

2. Materials and methods

2.1. RNA isolation and northern blot analyses

Total RNA was extracted with TRIzol (Gibco-BRL life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. For Northern blot analyses, 15–25 μg of total RNA was

* Corresponding author. Fax: +81-52-836-3455.
E-mail address: imagawa@phar.nagoya-cu.ac.jp (M. Imagawa).

Abbreviations: fad, factor for adipocyte differentiation; C/EBP, CCAAT/enhancer-binding protein; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxantine; ORF, open reading frame; PPARγ, peroxisome proliferator-activated receptor γ; RACE, rapid amplification of cDNA ends; RGS2, regulator of G protein signaling 2; RT-PCR, reverse transcriptase coupled-PCR; shRNA, short-hairpin RNA; SREBP, sterol regulatory element-binding protein; TCL/TC10βL, TC10-like/TC10βLong

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electrophoresed on a 1% agarose gel containing 2% formaldehyde and then transferred to a Hybond-N+ nylon membrane (Amersham Biosciences). Each probe was labeled with [α-^{32}P]dCTP using a BcaBEST labeling kit (Takara Biomedicals, Kusatsu, Japan).

2.2. Cloning of full-length cDNA of mouse fad104

Since mouse *fad*104 cDNA was isolated as a small 584 bp fragment, library screening, the 5′-rapid amplification of cDNA ends (5′-RACE) and the reverse transcriptase coupled-polymerase chain reaction (RT-PCR) were used for the cloning of full-length cDNA. Library screening was done using the mouse kidney λgt10 cDNA library (BD Biosciences CLONTECH). 5′-RACE was performed using a Marathon cDNA Amplification Kit (BD Biosciences CLONTECH) following the instructions of the manufacturer. Total RNA was prepared from 3T3-L1 cells 3 h after induction. mRNA was isolated from total RNA using oligotex-dT30 (Daiichi Pure Chemicals, Tokyo, Japan) according to the manufacturer's directions.

The single strand cDNA was amplified with oligo(dT) primer and AMV reverse transcriptase. The second strand cDNA was synthesized using a second-strand enzyme cocktail containing RNase H, *Escherichia coli* DNA polymerase I, and *E. coli* DNA ligase. The resultant double stranded cDNA was ligated to a Marathon cDNA adapter by T4 DNA ligase. The PCR was performed using the forward primer AP-1: 5′-CCATCCTAATACGACTCACTATAGGGC-3′ and a *fad*104-specific reverse primer-1: 5′-GCTTGCCCGCCGCTTCCTTCTCTTC-3′, and primer-2: 5′-GGTGCGCAGCTATGGGTGGTGAAGC-3′. The fragments obtained from 5′-RACE were subcloned into a T-added *Eco*RV site of pBluescript KS+.

2.3. DNA sequencing and database analysis

The sequence was determined with the automated sequencer DSQ-1000 (Shimadzu Corp., Kyoto, Japan) and an ABI PRISM 310 (Applied Biosystems, Foster City, CA). The human ortholog of *fad*104 was predicted using the Human Genome Database. Searches for the human ortholog in the Human Genome Database were performed using BLAST programs accessed via the NCBI homepage.

2.4. Cell culture and differentiation

Mouse 3T3-L1 (ATCC CL173) preadipocyte cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. For the differentiation experiment, the medium was replaced with DMEM containing 10% fetal bovine serum (FBS), 10 μg/ml of insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 μM dexamethasone (Dex) at 2 days post-confluence. After 2 days, the medium was changed to DMEM containing 5 μg/ml of insulin and 10% FBS, then the cells were refed every 2 days. Mouse 3T3-F442A (ECACC 70654) cells were maintained in DMEM containing 10% calf serum. For the differentiation experiment, the medium was replaced with DMEM containing 10% FBS and 5 μg/ml of insulin at confluent. The cells were refed every 2 days. Mouse NIH-3T3 (clone 5611, JCRB 0615) fibroblastic cells were maintained in DMEM containing 10% calf serum.

2.5. Fractionation of fat cells

The fat cells were prepared as described previously [13]. In brief, epidermal fat pads were isolated from male C57Bl/6J mice aged 6 weeks, washed with sterile PBS, minced, and washed with Krebs–Ringer bicarbonate (KRB) buffer (pH 7.4). Then, the minced tissue was digested with 1.5 mg/ml of collagenase type II (Sigma–Aldrich, Inc., St. Louis, MO) in KRB buffer, containing 4% bovine serum albumin at 37 °C for 1 h on a shaking platform. The undigested tissue was removed with a 250 μm nylon mesh and the digested fraction was centrifuged at $500 \times g$ for 5 min. The adipocytes were obtained from the most upper layer, washed with buffer, and centrifuged to remove other cells. The stromal-vascular cells were resuspended in the erythrocyte lysis buffer (150 mM NH_4Cl, 25 mM NH_4HCO_3 and 1 mM EDTA (pH 7.7)), filtered through 28 μm nylon mesh and then precipitated at $500 \times g$ for 5 min.

2.6. RNAi experiment

The five target regions (1: 160–178 bp; 2: 627–645 bp; 3: 1078–1096 bp; 4: 1813–1831 bp; and 5: 3391–4009 bp) were selected using the QIAGEN siRNA online design tool (http://sirna.qiagen.com/) for the RNAi experiment with *fad*104. A 19-nt short-hairpin RNA (shRNA)-coding fragment with a 5′-TTCAAGAGA-3′ loop was subcloned into the *Apa*I/*Eco*RI site of pSilencer 1.0-U6 (Ambion, Inc., Austin, TX). As a negative control, the scrambled fragment 5′-GTAAGATGAGGCAATGGAG-3′ which does not have similarity with any mRNA listed in GenBank was generated.

Transfection of shRNA-expressing vectors into 3T3-L1 cells was performed with Nucleofector (Amaxa, Cologne, Germany) using Cell Line Nucleofector Kit V (Amaxa). 3T3-L1 cells were harvested and resuspended in Nucleofector solution at 1.0×10^6 cells/100 μl. After addition of 9 μg of shRNA expression vectors, the cells were transfected using program 'T-20' of Nucleofector. Then, the cells were spread on 6- or 24-well plates. The transfected 3T3-L1 cells were subjected to differentiation experiments three days after the transfection. The differentiation experiments were done using the same induction medium described above.

2.7. Real-time quantitative RT-PCR (Q-PCR)

Isolation and reverse transcription of total RNA were done as described above. ABI PRISM 5700 sequence detection system (Applied Biosystems) was used to perform Q-PCR. The pre-designed primers and probe sets of *fad*104, PPARγ, C/EBPα, C/EBPβ, C/EBPδ, SREBP-1, LPL and 18S rRNA were obtained from Applied Biosystems. The reaction mixture was prepared using a TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The mixture was incubated at 50 °C for 2 min and at 95 °C for 10 min, and then the PCR was done at 95 °C for 15 s and at 60 °C for 1 min for 40 cycles. The relative standard curves were generated in each experiment to calculate the input amounts of the unknown samples.

3. Results

3.1. Expression of fad104 during the early stages of adipogenesis

We previously reported the isolation of 102 clones, which were expressed at 3 h after induction by the PCR-subtraction cloning method. Since BLAST searches revealed no significant matches against proteins of known function, 46 clones are thought to be novel genes [8,9]. To investigate the role of one of these genes, *fad*104, during adipocyte differentiation, we first determined the time course of *fad*104 expression during the early stages of adipogenesis of mouse 3T3-L1 cells by Northern blotting (Fig. 1A). While the expression of *fad*104 was almost undetectable before stimulation, it was quickly upregulated after the induction, and reached a peak at 3 h and then declined until 24 h after the induction. For exact quantification of the signal for *fad*104 mRNA, we performed Q-PCR for *fad*104 during the early and late stages of adipogenesis of 3T3-L1 cells (Fig. 1B). The expression level of *fad*104 in 3T3-L1 cells reached a peak at 3 h after induction. We also determined the expression level of *fad*104 in mouse 3T3-F442A cells, which are another preadipocytes and do not require IBMX and Dex for differentiation (Fig. 1C). Interestingly, *fad*104 expression reached a peak at 3 h as found in 3T3-L1 cells. These findings indicate that *fad*104 expresses transiently at the early stages of adipocyte differentiation.

3.2. Expression profile of fad104 in the adipocyte differentiable state and the non-differentiable state

Since the expression of *fad*104 was observed in the earliest stage of adipogenesis, we next compared the expression level of *fad*104 between the adipocyte differentiable state and non-differentiable state. It is well documented that 3T3-L1 cells undergo differentiation when stimulated at 2 days post-confluence (growth arrested), while proliferating 3T3-L1 cells do not differentiate in response to adipogenic inducers. Another mouse fibroblast cell line, NIH-3T3, is non-adipogenic and

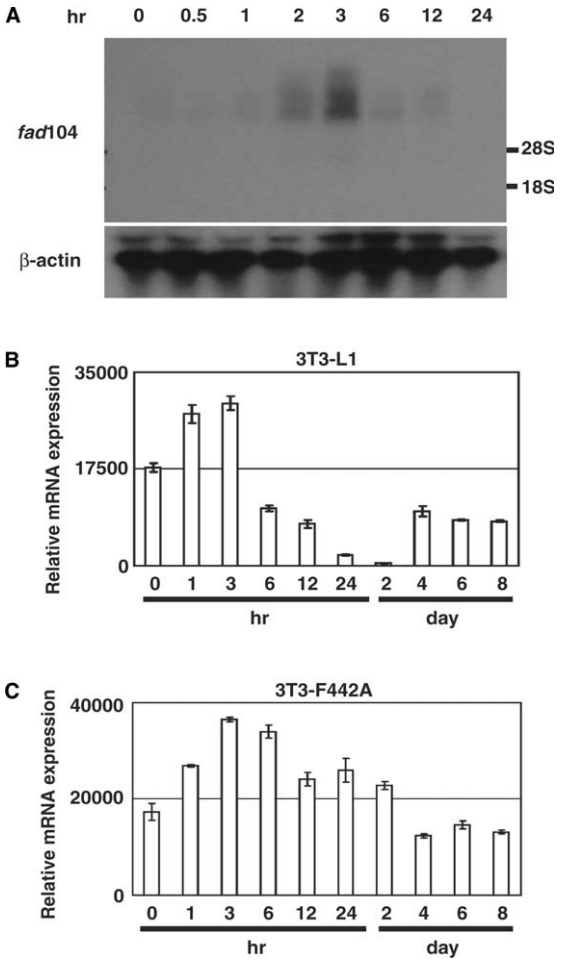


Fig. 1. Time course of *fad*104 mRNA expression in the early stages of adipocyte differentiation. (A) Northern blot analysis of *fad*104 expression in 3T3-L1 cells. Total RNA from different time points after the induction was prepared from 3T3-L1 cells. Isolated total RNA (15 μg) was loaded and subjected to Northern blot analysis of *fad*104. β-Actin as a control is also shown. (B) Q-PCR analysis of *fad*104 expression in 3T3-L1 cells. The expression level of *fad*104 was determined at various time points of adipocyte differentiation of 3T3-L1 cells by Q-PCR and normalized with 18S rRNA expression determined by Q-PCR. Each column represents the mean with standard deviation ($n = 3$). (C) Q-PCR analysis of *fad*104 expression in 3T3-F442A cells. The expression level of *fad*104 was determined at various time points of adipocyte differentiation of 3T3-F442A cells by Q-PCR and normalized with 18S rRNA expression determined by Q-PCR. Each column represents the mean with standard deviation ($n = 3$).

does not differentiate into adipocyte in either a growth arrested or proliferating state. These two cell lines were stimulated with inducers for 3 h. Total RNA was prepared from non-stimulated cells (0 h) and from cells at 3 h after stimulation. Expression of *fad*104 was only elevated in the growth-arrested 3T3-L1 cells, its expression was not observed in the other three states (Fig. 2). These results indicate that the expression of *fad*104 is strongly restricted to the adipocyte differentiable state.

3.3. Cloning of full-length mouse fad104 cDNA

To prevent bias, amplified cDNA fragments isolated by the PCR-subtraction cloning method were digested into 300–500 bp fragments by *Rsa*I [8]. Consequently, the *fad*104 cDNA fragment obtained by this cloning method was 584 bp long.

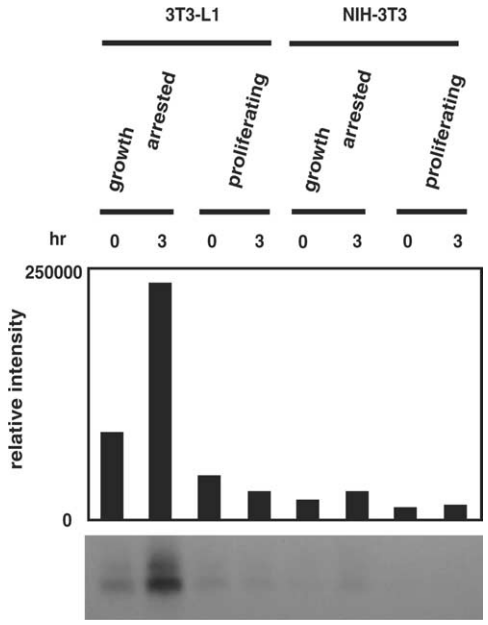


Fig. 2. Expression profile of *fad*104 in the adipocyte differentiating and non-differentiating cells. Total RNA was isolated from proliferating and post-confluent 3T3-L1 and NIH-3T3 cells, before and 3 h after induction. A 25 μg amount of isolated total RNA is loaded in each column. Relative intensity is also shown.

Therefore, we next isolated the full-length cDNA of *fad*104 using library screening and 5′-RACE as shown in Fig. 3A. Mouse kidney library screening using the subtracted *fad*104 fragment as a probe enabled us to isolate a 1606 bp fragment

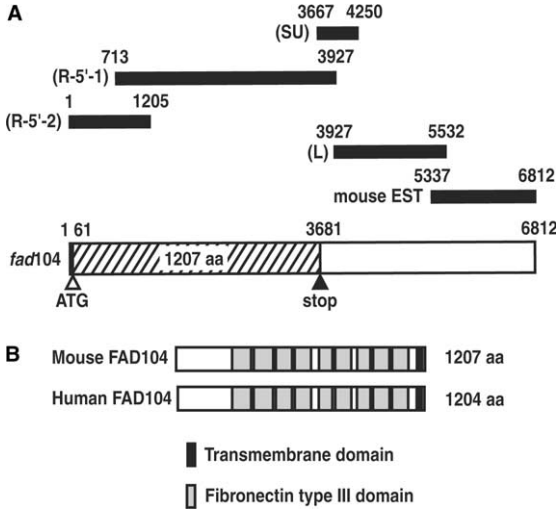


Fig. 3. Cloning of mouse *fad*104 and the deduced amino acid structure of mouse and human FAD104. (A) The full-length cDNA for mouse *fad*104 was isolated by 5′-RACE and library screening. Su, R-5′-1, R-5′-2, and L indicate the fragments obtained from the original subtraction, 5′-RACE, and library screening, respectively. The combined sequence is shown as *fad*104 and start and stop codons are indicated. The predicted number of amino acids for mouse FAD104 is 1207. (B) The schematic representation of mouse and human FAD104. The deduced amino acid sequences encoded by the 3621 bp ORF of mouse *fad*104 and 3612 bp ORF of human *fad*104. The nine repeats of the fibronectin type III domain (according to NCBI RPS-BLAST) and single transmembrane domain (according to the SOSUI system) are shown.

(L in Fig. 3A). A database analysis using this 1606 bp fragment identified a mouse EST that shows 100% similarity with *fad*104 and contains a poly A additional signal at the 3′ terminal.

We next performed 5′-RACE using cDNA prepared from 3T3-L1 cells 3 h after induction and isolated a 3215 bp cDNA fragment with the first 5′-RACE (R-5′-1 in Fig. 3A). In the second 5′-RACE, a 1205 bp cDNA fragment including a 712 bp unidentified region was obtained (R-5′-2 in Fig. 3A). Finally, the combined sequences of the subtracted fragment and fragments obtained by the 5′-RACE, library screening, and database search resulted in a full-length 6812 bp cDNA fragment with an open reading frame (ORF) of 1207 amino acids (GenBank Accession No. AB098596).

3.4. Sequence analyses of mouse and human fad104

We attempted to predict the human ortholog cDNA of *fad*104 by making a BLAST search of the Human Genome Database [14] using the mouse *fad*104 sequence. The search indicated a genomic fragment located at locus 3q26 + 3 of human chromosome 3 and comprising 25 exons and 24 introns. In all the exon/intron junctions, the GT/AG rule was conserved (data not shown). By splicing out the introns and combining the exons, the human ortholog of *fad*104 was predicted. The human ortholog of *fad*104 was a 6894 bp cDNA containing a 3612 bp ORF encoding 1204 amino acids (GenBank Accession No. AB098597). In both mouse and human *fad*104, an ATG at position 61, existing at the 5′-end within the coding region of the cDNA, is likely to serve as the start codon for translation initiation, although the sequence flanking this ATG does not conform very well to Kozak's rule [15].

A BLAST search of the Conserved Domain Database using the deduced amino acid sequences of mouse and human FAD104 revealed the existence of 9 repeats of the fibronectin type III domain. A search with the SOSUI system showed that a single transmembrane domain was present in the C-terminal end of the mouse and human FAD104 (Fig. 3B).

3.5. Genomic distribution of mouse fad104

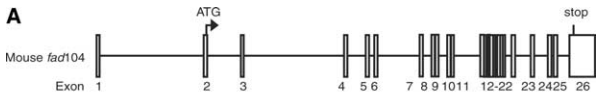
The genomic distribution of *fad*104 was identified by a BLAST search of The Mouse Genome Database, which was made public by The Mouse Genome Sequencing Consortium [16]. The search indicated that mouse *fad*104 was located at 3A3 of mouse chromosome 3 and constituted 26 exons and 25 introns. In the sequences of the exon/intron junctions, the GT/AG rule was conserved in all cases (Fig. 4).

3.6. Expression of fad104 in various mouse tissues

To examine the tissue distribution of *fad*104, Q-PCR was performed for *fad*104 against brain, heart, skeletal muscle, kidney, lung, liver, testis, white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) isolated from adult mouse. WAT was fractionated into stromal-vascular cells and adipocytes. Interestingly, the expression of *fad*104 was found to be highly WAT selective and the level of expression in other organs including BAT was relatively low. Moreover, its expression was higher in the stromal-vascular cells, indicating that the expression of *fad*104 is predominant in preadipocytes (Fig. 5).

3.7. RNAi experiment on fad104 in 3T3-L1 cells

As described above, the transient expression of mouse *fad*104 was observed at an early stage in the differentiation of 3T3-L1 cells. Furthermore, its expression was strongly restricted to cells in a differentiable state. However, the function of *fad*104 in adipocyte differentiation remains unknown. For



B Intron/exon junction sequence of mouse *fad*104 gene

Exon	3′-splice acceptor	5′-splice donor	Exon size (bp)
1		CGGAGCGCGG **gt**cagtcggt	32
2	ttgcaggt**ag** CAGGTTGAGG	AGCTCAGCAG **gt**cggtgagc	135
3	gttgtcac**ag** GTCATTCTTG	TGCATCCAAG **gt**aaggatgt	76
4	tatctttc**ag** GACCTGCTGA	TATCTCACAG **gt**atggcatt	77
5	tcttccct**ag** GTGATTGAAG	AGCGAGCCAG **gt**aagtaggt	244
6	ttcattct**ag** AAATTATCCC	GACCTGCAAG **gt**aatggcat	291
7	tgcaatac**ag** AGTATGAGTT	GAAACCACAG **gt**atgtcttc	59
8	cttttttc**ag** GTGTCTAATA	TCATTTACAG **gt**ggagtgtg	152
9	tgttttcc**ag** TGGAGAAGAG	ACCATGTGAG **gt**gagttagc	60
10	tcctttgc**ag** GGTATATGCC	GCAGTGGAAG **gt**gggtagtt	139
11	cttttttt**ag** GCTCCAATTG	ATGGGATGAG **gt**aagcctcc	54
12	gtccttcc**ag** GGAAAGAGAA	TCGGCACCAG **gt**atggcgtc	125
13	tcccccac**ag** TGGTTACAGC	GGATGAGAAC **gt**gagtccca	175
14	gcctcttc**ag** GATAGCCATT	TAAATTCAGG **gt**gagtcttc	187
15	tgctctgc**ag** CTGACTGCTT	GTCAAGTGGG **gt**atgtctcc	139
16	tactctgc**ag** ATGCTCCAAA	ACTTCTGAAG **gt**gacacttt	72
17	cattttcc**ag** CTGGGCAGTG	GCACAGTCAG **gt**tggttcat	119
18	tctcccac**ag** TGTTCTGAAA	TTAGAGTGGG **gt**gagtgaac	106
19	acatcctc**ag** ATGTGCCTGC	TGACGGAGGG **gt**gagtatga	173
20	tctctttc**ag** TACGGTCCCT	GGGCTGGGAG **gt**gagtatgg	114
21	tgccctgc**ag** AGCCCTGCGA	CAGACTACAG **gt**aggtgagc	150
22	ttttttcc**ag** GCCTTCAATC	CGACATACCG **gt**gagtggaa	281
23	ccacctct**ag** GATCAGAATT	GGAACAAGAG **gt**gagacggg	213
24	tctctctt**ag** GTTTATCTCA	ACCCTCAAAG **gt**gtgtagag	167
25	tgtattgc**ag** CACCTCGAGT	GTACAAGCAG **gt**aaggacca	128
26	ttctttga**ag** GTGTACAAGG		3344

Fig. 4. Genomic structure of mouse *fad*104 obtained from the Mouse Genome Database. (A) The exon–intron structure of mouse *fad*104 predicted from the Mouse Genome Database. (B) The nucleotide sequences at the exon–intron junctions of mouse *fad*104. The sequences in uppercase and lowercase letters are those of exons and introns, respectively. The GT/AG nucleotides at the beginning and end of the introns are shown in boldface.

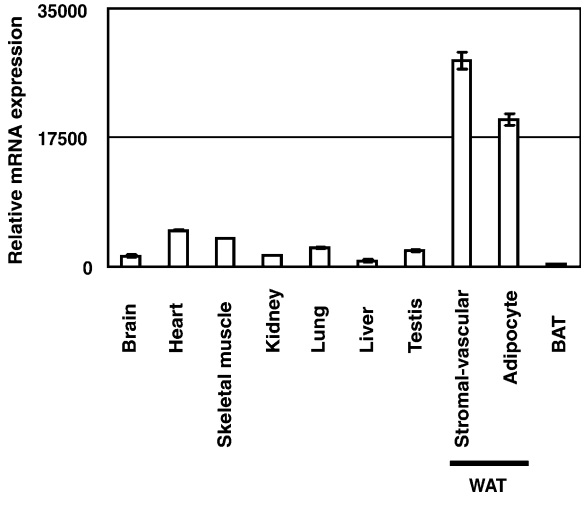


Fig. 5. Tissue distribution of *fad*104. The expression level of *fad*104 in various tissues isolated from C57Bl/6J mice was determined by Q-PCR and normalized with 18S rRNA expression determined by Q-PCR. Stromal-vascular cells and adipocytes were fractionated from isolated white adipose tissue. Each column represents the mean with standard deviation ($n = 3$). WAT, white adipose tissue; BAT, brown adipose tissue.

the functional analysis of *fad*104 during adipogenesis, we have conducted an RNAi experiment to knock down the expression of *fad*104 in adipogenesis. Five shRNA expression vectors with different target regions were introduced into 3T3-L1 cells. Three days after the transfection, cells were stimulated with adipogenic inducers. Three hours after the induction, total RNA was isolated and expression levels of *fad*104 were identified by RT-PCR. Of the five target regions, region 4 showed the greatest reduction in activity (data not shown). Therefore, we used the region 4-shRNA expression vector for further analyses.

First, we performed RT-PCR to detect *fad*104 mRNA and found that the expression was knocked down. However, since RT-PCR is semi-quantitative, we next confirmed the expression level of *fad*104 by Northern blot analysis. As shown in Fig. 6A, *fad*104 expression in shRNA expression vector-transfected cells had declined compared with that in the control cells. Using these transfected cells, we conducted a differentiation experiment. Three days after the transfection, the cells were treated with inducers. After 8 days of induction, the cells were fixed and stained with Oil red O (Fig. 6B) and the amounts of triacylglycerol were determined (Fig. 6C). The number of Oil red O-stained cells was decreased in the shRNA expression vector-transfected cells and the accumulation of triacylglycerol was also reduced. These results imply that the decrease in *fad*104 expression reduces the ability of 3T3-L1 cells to differentiate.

Next, we determined the expression profiles of adipogenic marker genes by Q-PCR (Fig. 6D). During the differentiation of si*fad*104 cells, the expression levels of PPARγ, C/EBPα, SREBP-1 and LPL were declined when compared with those in the control cells. These results strongly indicate that the reduction of *fad*104 expression inhibits the adipogenesis of 3T3-L1 cells. Interestingly, the expression levels of C/EBPβ and C/EBPδ were unchanged.

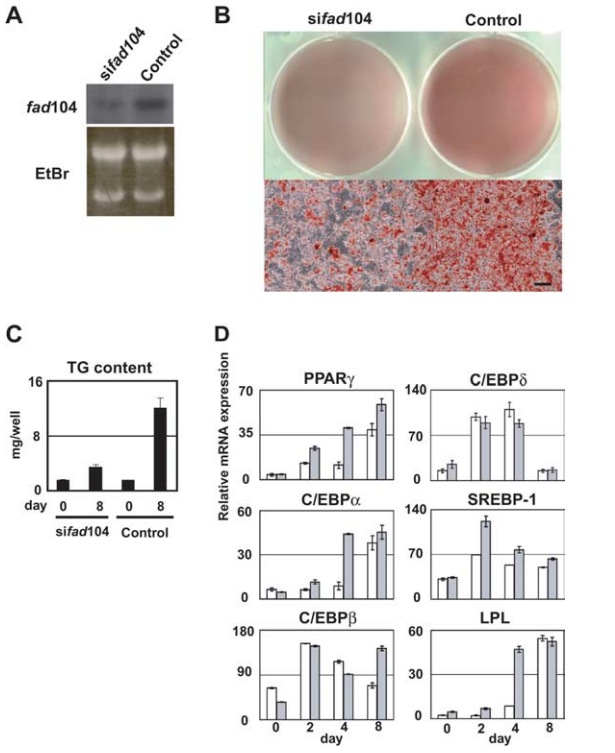


Fig. 6. The functional analysis of *fad*104 by RNAi. (A) The endogenous expression of the *fad*104 gene was determined by Northern blot analysis. Total RNA (15 μg) obtained from 3T3-L1 cells transfected with shRNA expression vector for *fad*104 (si*fad*104) or with scrambled shRNA expression vector as a control (Control) was subjected to Northern blot analysis of *fad*104. Staining with ethidium bromide (EtBr) for ribosomal RNA is shown as a control. (B) Differentiation of 3T3-L1 cells transfected with shRNA expression vector for *fad*104 (si*fad*104) or with scrambled shRNA expression vector as a control (Control) was stimulated with inducers. After 8 days, the cells were fixed and stained with Oil red O to detect oil droplets. Bar, 200 μm. Staining was done on 6-well plates. (C) The amounts of triacylglycerol were measured. The measurement of triacylglycerol content was done on 24-well plates. The column shows the mean with standard deviation ($n = 3$). (D) Effect of shRNA expression for *fad*104 on the expression of various adipogenic genes. Total RNA obtained from si*fad*104 cells (white bar) or Control (gray bar) at each time point was subjected to Q-PCR. Expression level was normalized with 18S rRNA expression determined by Q-PCR. Each column represents the mean with standard deviation ($n = 3$).

4. Discussion

The PPARγ and C/EBP families have been implicated in adipocyte differentiation as master regulators. PPARγ and C/EBPα were demonstrated to have critical roles during adipocyte differentiation in a gain of function experiment [3,4]. The knocking out of PPARγ resulted in mice that failed to develop adipocytes [17]. The same result was also obtained on the knocking out of C/EBPα [18]. The expression of C/EBPβ and C/EBPδ is observed earlier than that of PPARγ and C/EBPα [19]. It is shown that coexpression of C/EBPβ and C/EBPδ contributes to the expression of PPARγ and C/EBPα [6,20]. Double knockout of C/EBPβ and C/EBPδ disrupted the formation of adipose tissue in mice. However, PPARγ and C/EBPα were normally expressed [21]. Moreover, the genes expressed prior to C/EBPβ and C/EBPδ during adipocyte differentiation remain largely unknown. These results indicate that an unknown cascade underlies adipogenesis, especially in the early stages.

In our laboratory, we have been focusing on the earliest steps of differentiation, since the initiation of adipogenesis is poorly understood as described above. We have already identified RGS2, TCL/TC10βL and p68 RNA helicase as factors accelerating adipogenesis which are expressed at the very beginning of differentiation [10–12]. In the present study, we have cloned and characterized *fad*104. *Fad*104 was one of the 46 unknown genes included in the cDNA pool of 102 genes isolated by the PCR-subtraction cloning method [8,9]. Expression of *fad*104 was observed 3 h after the stimulation of mouse 3T3-L1 cells and 3T3-F442A cells with adipogenic inducers. Mouse 3T3-L1 cells do not differentiate into adipocytes when kept in a proliferating state. Mouse NIH-3T3 fibroblasts do not differentiate into adipocytes either even in a growth-arrested state. Since the expression of *fad*104 was only observed in growth-arrested 3T3-L1 cells, it was restricted to the adipocyte differentiable cells.

Owing to the interesting expression pattern of *fad*104 during adipogenesis, we attempt to isolate full-length cDNA of *fad*104. *Fad*104 was characterized as a 6812 bp cDNA with an ORF of 3621 bp encoding 1207 amino acids. Prediction of the *fad*104 human homolog was done by searching the Human

Genome Database. The predicted human ortholog was 6894 bp long containing a 3612 bp ORF of 1204 amino acids. The human ortholog of FAD104 was three amino acids shorter than its mouse counterpart. Interestingly, the mouse and human amino acid sequences were highly conserved with 92.5% similarity. A BLAST search of the Conserved Domain Database and the SOSUI system indicated that FAD104 has nine fibronectin type III domains and a single transmembrane domain in both mouse and human.

Using another transmembrane prediction system 'TMHMM', FAD104 is found to have only three amino acids in inner membrane region. Preadipocyte factor 1 (pref-1) has a single transmembrane like FAD104 and functions as a negative regulator of adipocyte differentiation. Interestingly, it is reported that this protein is processed at the membrane-proximal processing site and functions as a soluble protein [22]. Therefore, we have searched for the same cleavage site in FAD104 and checked the possibility of FAD104 as a soluble protein by proteolytic maturation. However, the cleavage site of pref-1 was not conserved in FAD104. A possible link between the preadipocytes and the extracellular matrix to regulate adipose conversion remains to be elucidated. The eighth fibronectin repeat of mouse *fad*104 contained an arginine–glycine–aspartic acid (RGD) tripeptide sequence, which is a potential cell-binding domain via integrin [23]. The fibronectin type III domain is found in proteins like cell adhesion molecules and cell surface receptors.

The tissue distribution analysis revealed that *fad*104 was predominantly expressed in WAT, especially, highly expressed in the stromal-vascular cells than in adipocytes. Therefore, we next performed a functional analysis of *fad*104 using the RNAi method. When the expression of *fad*104 was downregulated during the initiation of adipogenesis of 3T3-L1 cells, the accumulation of oil was suppressed, indicating that *fad*104 has an important role in adipogenesis. Furthermore, the expression levels of PPARγ, C/EBPα and SREBP-1 as well as LPL were declined, while those of C/EBPβ and C/EBPδ did not change. These results indicate that *fad*104 affects the gene expression at the mid-phase of adipocyte differentiation and may contribute to the signaling pathway at this stage.

It is likely that the inhibition of *fad*104 expression does not cause a delay in differentiation, rather inhibition of differentiation ability of 3T3-L1 cells. We have checked the differentiation ability of si*fad*104 cells and control cells very carefully. The differentiation occurred at day 4 in both cells and the differentiation reached to a maximum at around 6–8 days. Throughout this period, the percentage of differentiated cells in si*fad*104 cells is less than that in control cells (data not shown). However, a further characterization is definitely needed and the detailed analyses at a stage of mitotic clonal expansion should also be elucidated.

Fibronectin is one of the extracellular matrix proteins and functions as a ligand of integrin. The interaction of integrin with extracellular ligand leads to the organization of an actin-containing cytoskeleton and signal transduction [24]. It is also reported that differentiation of 3T3-F442A preadipocytes was inhibited when induced on the fibronectin matrix [25]. This result indicates the importance of interaction between differentiating cells and the extracellular matrix. It is interesting that the gene for a fibronectin type III-containing protein, *fad*104, is transiently expressed during adipocyte differentiation accompanied by major morphological changes. However, the biochemical function of *fad*104 remains unknown. Moreover, it is unclear whether the results obtained here are conserved *in vivo*. To elucidate these important points, the development of knockout mice is now in progress.

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