Biochemical and Biophysical Research Communications 440 (2013) 521–526

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel link between *Slc22a18* and fat accumulation revealed by a mutation in the spontaneously hypertensive rat

Takashi Yamamoto [a,b], Kozue Izumi-Yamamoto [a,b], Yoko Iizuka [c], Midori Shirota [a,b], Miki Nagase [b], Toshiro Fujita [d], Takanari Gotoda [a,b,*]

[a] Department of Clinical and Molecular Epidemiology, 22nd Century Medical and Research Center, The University of Tokyo, Tokyo 113-8655, Japan
[b] Department of Nephrology and Endocrinology, The University of Tokyo, Tokyo 113-8655, Japan
[c] Department of Diabetes and Metabolic Disease, The University of Tokyo, Tokyo 113-8655, Japan
[d] Division of Clinical Epigenetics, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 153-8904, Japan

ARTICLE INFO

Article history:
Received 15 September 2013
Available online 4 October 2013

Keywords:
SHR
QTL
Cd36
Solute carrier
Adipocyte differentiation
Obesity

ABSTRACT

Two different strains of the spontaneously hypertensive rat (SHR) exist, either with or without a *Cd36* mutation. In the F2 population derived from a cross between these two SHR strains, the mutant *Cd36* allele was tightly linked to differences in metabolic phenotypes but not to those in fat pad weight. This suggested the existence of another crucial mutation related to adiposity. Linkage analysis of this F2 population showed a significant linkage between the rat chromosome 1 region (*D1Rat240–D1Wox28*) and fat pad weight. By integrating both positional and expression information, we identified a donor splice site mutation in the gene for solute carrier family 22 member 18 (Slc22a18) in SHR with reduced fat pad weight. This mutation was located at the linkage peak with a maximum logarithm of odds score of 7.7 and caused skipping of the whole exon 9 that results in a complete loss of a whole membrane-spanning region of the rat Slc22a18 protein. *Slc22a18* mRNA was abundantly expressed in isolated adipocytes and in a differentiation-dependent manner in 3T3-L1 cells. Knockdown of the *Slc22a18* mRNA via infection of adenoviral vectors markedly inhibited both triglyceride accumulation and adipocyte differentiation in 3T3-L1 cells. By contrast, overexpression of the *Slc22a18* mRNA had the opposite effects. These results reveal a novel link between *Slc22a18* and fat accumulation and suggest that this gene could be a new therapeutic target in obesity.

1. Introduction

The spontaneously hypertensive rat (SHR) is a widely-used animal model of essential hypertension and demonstrates a series of manifestations of insulin resistance syndrome [1]. We previously reported that there were two different SHR strains, depending on the presence or absence of a *de novo* mutation in the gene for Cd36, and that they manifested significant differences in several important phenotypes [2]. The SHR strain with a *Cd36* null mutation (hereafter referred to as SHR/NCrj) had significantly reduced blood glucose and triglyceride levels and increased blood free fatty acid levels as compared with the SHR strain without this mutation (referred to as SHR/Izm). Furthermore, the SHR/NCrj strain also showed markedly decreased epididymal fat pad weight as compared with the SHR/Izm strain, although both SHR strains had comparable body weights [2].

Cd36 is a multi-functional transporter that is involved in glucose and lipid metabolism and is known to facilitate the uptake of long-chain fatty acids in adipocytes [3]. Taking into consideration the known function of Cd36 and the extreme genetic similarity between these two SHR strains, we hypothesised that the phenotypic differences observed between these two strains could be attributable to a *Cd36* mutation.

In the present study, we initially examined for possible linkages between the mutant *Cd36* allele and the respective phenotypes in an F2 population derived from a cross between these SHR strains. Comparing these relatively identical strains provides a unique opportunity for linkage analysis with minimal effects of genetic noise. In this F2 cross, as expected, the mutant *Cd36* allele was significantly linked to altered metabolic phenotypes, but unexpectedly, not to the differences in fat pad weight, which strongly suggested another crucial mutation underling the differences in adiposity between these two rat strains. Based on the results of linkage and mRNA expression analyses, we report a novel link between solute carrier family 22 member 18 (Slc22a18) and fat accumulation.

* Corresponding author at: Department of Clinical and Molecular Epidemiology, 22nd Century Medical and Research Center, The University of Tokyo Hospital, 7-3-1 Hongo, Tokyo 113-8655, Japan. Fax: +81 3 5800 9169.
E-mail address: gotoda-tky@umin.ac.jp (T. Gotoda).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.09.096

2. Materials and methods

2.1. Animals

NCrj rat strains were purchased from Charles River (Japan) and Izm strains were from Funabashi Farm (Chiba, Japan). All rats were maintained under stable conditions on a 12-h light–dark cycle and fed standard laboratory chow (MF purchased from Oriental Yeast, Ltd., Japan) and water ad libitum. At 12 weeks of age, after measuring its body weight, a male rat was sacrificed after an overnight fast. Arterial blood and tissue samples were collected rapidly, frosen in liquid nitrogen, and kept at −80 °C prior to analyses. Whole blood glucose levels were measured using a glucometer (Sankyo, Japan), and serum cholesterol and free fatty acid levels were determined by enzymatic assays (Kyowa, Japan). All animal procedures were performed in accordance with the guidelines for the care and use of laboratory animals approved by University of Tokyo Graduate School of Medicine.

2.2. Isolation of genomic DNA, genotyping, and DNA sequencing

DNA was isolated from a whole blood sample of each rat with a DNA extractor WB kit (WAKO, Japan). DNA (100 ng) was used for polymerase chain reaction (PCR) as previously described [4]. The resulting PCR products were analysed with 1–4% NuSieve 3:1 agarose gels (Takara Bio, Japan) or with a GenePhor DNA Separation System (GE Healthcare) for single strand conformation polymorphism (SSCP) analysis. A polymorphic (CA)*n*-repeat site was found 13-bases downstream of the *Slc22a18* mutation site, and tight linkage was observed between the wild-type G allele and $(CA)_{18}$ and between the mutant A allele and $(CA)_{28}$ (Supplementary Table 1). PCR was run with a pair of primers (upstream: 5′-CTGCTGAGATCCAGTGTACT-3′, downstream: 5′-TGGAGGATGGCTTGAGACCT-3′) that spanned both the mutation and (CA)*n*-repeat sites, which generated PCR-products of 142 base-pairs (bp) for the wild-type allele and of 162 bp for the mutant allele. Genotyping of the *Cd36* mutation was performed as previously described [2]. Genotyping for 11 other polymorphic microsatellite markers (*D1Wox32*, *D1Rat240*, *D1Wox28*, *D1Got209*, *D1Wox10*, *D1Rat169*, *D1Rat75*, *D1Rat77*, *D1Mit7*, *D1Rat119*, *D1Wox25*) was performed according to recommended conditions available in public rat databases. Direct DNA sequencing of the PCR-amplified products was performed as described [4].

2.3. Isolation of RNA, northern blot and microarray analyses, and real-time reverse-transcription PCR (RT-PCR)

Total RNA was extracted from tissues or cells using 1 ml of TRIzol reagent (Invitrogen). The purity and concentration of each RNA sample were determined by measuring the absorbance at 260/280 nm. To further determine the quality of RNA, 1 μg of total RNA was run on a 1% agarose gel to inspect the quality of the 28 S and 18 S ribosomal bands. cDNA was synthesised using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. A full-length cDNA probe for rat *Slc22a18* mRNA was prepared by cloning its RT-PCR product from rat liver into pGEM-T easy vectors (Promega) and its authenticity was confirmed by DNA sequencing. Total RNA (10 μg) was electrophoresed on a 1% agarose gel containing formaldehyde, and then transferred to a nylon membrane, Hybond N (Amersham Biosciences). Membranes were hybridised with [^{32}P] dCTP-labeled *Slc22a18* cDNA probes using a Megaprime DNA labeling kit in rapid-hyb buffer (Amersham Biosciences). After washing with 0.1× SSC, 0.1% SDS at 65 °C, the membranes were exposed to Kodak XAR-5 films (Kodak). Total RNA from rat epididymal adipose tissue was also subjected to an Oligotex mRNA purification kit (Takara Bio, Japan) and then analysed with a Rat Genome 230 2.0 Array (Affymetrix), which detected 20,861 rat genes expressed in adipose tissues. Gene expression was quantitatively analysed using real-time RT-PCR with LightCycler 480 SYBR Green I Master or LightCycler 480 Probes Master on a LightCycler 480 System II (Roche) as described previously [5]. DNA sequences of primers and probes that were used are listed in Supplementary Table 2. Messenger RNA of *36B4* was used as an RNA loading control for northern blot experiments and as an internal control in real-time RT-PCR.

2.4. Western blot analysis

A rabbit polyclonal antibody against rat Slc22a18 was generated by immunizing a rabbit with a synthetic peptide (C + KPLSQKGDKAR) for the C-terminal region of rat Slc22a18. Anti-mouse Slc22a18 antibody was also generated against a synthetic peptide (C + KPLSQKGEKAR) for its C-terminus. Anti V5-horseradish peroxidase (HRP)-conjugated antibody was obtained from Invitrogen, and antibodies against PPARγ and α-tubulin from Santa Cruz Biotechnology. Western blot analysis was performed as previously described [5] with some modifications. Whole cell lysates were prepared using lysis buffer (25 mM HEPES (pH7.9), 50 mM KCl, 6% glycerol, 5 mM EDTA, 5 mM $MgCl_2$, 1% TritonX100, 1 mM DTT, protease inhibitor cocktail (Roche)). Protein samples (50 μg) were subjected to 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) or native–PAGE without SDS. Proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare), immunoblotted with appropriate antibodies, and visualised with an ImageQuant LAS 4000 mini (GE Healthcare).

2.5. Isolation of rat primary adipocytes and 3T3-L1 cell culture

Male rat epididymal fat pads were excised and minced in PBS with 0.5% BSA. Collagenase (Sigma–Aldrich) was added at 1 mg/ml before incubation at 37 °C for 2 h with shaking. Suspensions were centrifuged at 200*g* for 1 min to remove cellular debris and oil. Precipitated materials were resuspended as a vascular stromal fraction (VSF). Total RNA of floating adipocytes and the VSF were extracted separately with TRIzol reagent and subjected to RT-PCR. Mouse 3T3-L1 cells were maintained and differentiated in DMEM (WAKO, Japan) with 10% FBS as previously described [5]. On Day 0, 3T3-L1 preadipocytes were stimulated with a mixture comprising insulin, dexamethasone (DEXA) and 3-isobutyl-1-methylxanthine (IBMX) (all from Sigma), which induced the differentiation of preadipocytes into mature adipocytes. Oil Red O staining of 3T3-L1 cells was performed as previously described [5].

2.6. Adenoviral expression

Two independent constructs for short hairpin RNA (shRNA) targeting of *Slc22a18* were subcloned into the U6 entry vector using primer sequences specific for mouse *Slc22a18* cDNA (#1: 5′-gtgtaaccgttgacctgaaacgtgtgctgtccgtttcgggtcaatggttgcac-3′, #2: 5′-gggtcattatactcatctaacgtgtgctgtccgttaggtgagtatgatgatcc-3′) to generate adenoviral vectors by homologous recombination with the pAd promoterless vector (Invitrogen). Recombinant adenovirus was produced in 293A cells and purified as previously described [6]. The titer of adenovirus was determined using Adeno-X Rapid Titer kit (BD Biosciences). Four days before differentiation, 3T3-L1 cells were infected with adenoviral vectors for expressing shRNA (Ad-shSlc#1, Ad-shSlc#2, or Ad-shLacZ(control)) at the multiplicity of infection (MOI) of 30. Adenoviruses expressing GFP (Ad-GFP) or V5-tagged mouse Slc22a18 (Ad-Slc22a18V5) were also generated using the pAd/CMV/V5-DEST

vector system (Invitrogen) according to the manufacturer's instructions.

2.7. Data analysis and statistical analysis

Inter-strain differences were evaluated by the unpaired, two-tailed Student's *t* test. Comparison among three groups was performed using one-way ANOVA. *P*-values of <0.05 were considered significant. Logarithm of odds (LOD) scores in quantitative trait locus (QTL) analysis and marker locations were estimated using WinQTLCartographer ver.2.5 [7]. QTL plots were drawn based on the results of interval mapping analysis. The estimated threshold for a LOD score used as evidence of significant linkage in the F2 population was 2.4 (significance level = 0.05, permutation times = 1000).

3. Results

3.1. Decreased fat pad weight is linked to rat chromosome 1 (D1Rat240–D1Wox28) and not to Cd36

Initially, we examined for possible linkages between the mutant *Cd36* allele and each phenotype of the F2 population ($n = 144$) derived from a cross between the two different SHR strains. In this F2 cross, the mutant *Cd36* allele was significantly linked to altered metabolic phenotypes on the genetic background of these SHR strains. However, the difference in fat pad weight was not linked to the *Cd36* mutation, which indicated the possibility of another crucial mutation(s) underlying the differences in fat pad weight (Fig. 1A).

To explore this additional mutation, we continued our linkage analysis on this F2 population. The merit of this analysis was that it minimised the genetic noise derived from other genes, while a drawback was that there were few known genetic markers that could discriminate between these two SHR strains. After testing more than 500 candidate genetic markers selected from the public databases, we found 25 markers that could discriminate between them by agarose gel analysis or by SSCP analysis on polyacrylamide gels. In addition, 14 of these 25 polymorphic markers were located on rat chromosome 1 (Supplementary Table 3), which suggested that a different gross segment on rat chromosome 1 had possibly been separately inherited in these strains.

We then determined the genotypes using 11 markers located within about an 80 Mbp-segment flanked by *D1Wox32* and *D1Wox25* in all the F2 rats and performed a linkage analysis. These results showed significant linkage of the rat chromosome 1 region (*D1Rat240–D1Wox28*) with fat pad weight or fat pad weight adjusted for body weight with a maximum LOD score of 4.4 downstream of *D1Wox32* by 17.3 cM (Fig. 1B). It is worth noting that there was no linkage with body weight itself.

3.2. Identification of a splicing mutation in Slc22a18 at the linkage peak for reduced adiposity

We then compared the mRNA expression profiles in rat adipose tissues by microarray analysis. We selected genes that had considerable mRNA expression levels in adipose tissues or that had expression levels that were considerably different between the two SHR strains. By integrating both the positional and expression information, we focused on and comparatively sequenced 33 candidate genes (Supplementary Table 4). Among these, we found a biologically meaningful change only in the gene for solute carrier family 22 member 18 (Slc22a18). DNA sequence comparisons revealed that the SHR/NCrj strain, but not the SHR/Izm strain, had a G-to-A point mutation in the donor splice site of intron 9 (Fig. 2A). Northern blot analysis of mRNA derived from liver and kidney, two principal organs with *Slc22a18* mRNA expression, demonstrated slightly shorter bands in samples from strain SHR/NCrj as compared with those from strain SHR/Izm (Fig. 2B). RT-PCR analysis using primers located on exons 6 and 11 produced differentially-migrating bands (Fig. 2C). These were subjected to comparative DNA sequencing, which demonstrated that the mutation resulted in complete skipping of the whole exon 9 in the cDNA (Fig. 2D).

This skipping of exon 9 (102 bp) should have resulted in an in-frame deletion of 34 amino acids of rat Slc22a18 that comprises 412 amino acids [8] and is suggested to be a transporter with 10 membrane-spanning regions [9]. This in-frame deletion could cause a marked structural change, including a complete loss of the whole of the 8th membrane-spanning region. Western blot analysis confirmed these differentially-migrating bands at the protein level (Fig. 3A). Inclusion of this mutation increased the maximum LOD score up to 7.7 at the peak point downstream of *D1Wox32* by 17.3 cM, the exact location of the rat *Slc22a18* gene (Fig. 3B).

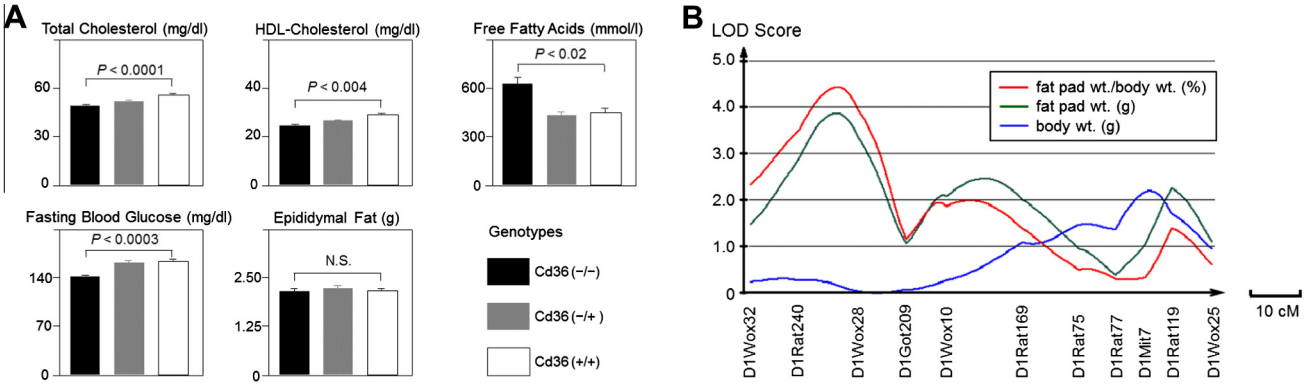

Fig. 1. Decreased epididymal fat pad weight is linked to a region on rat chromosome 1 and not to *Cd36*. (A) Linkage between the *Cd36* mutant allele and each phenotype was examined for F2 rats derived from a cross between strains SHR/NCrj and SHR/Izm (males, $n = 144$). Cd36(−/−) represents homozygous for the mutant *Cd36* allele, and Cd36(−/+) and Cd36(+/+) represent heterozygous and homozygous for the wild-type allele, respectively. (B) QTL plot for a gross segment of rat chromosome 1 (*D1Wox32–D1Wox25*) for epididymal fat pad weight (wt.), body wt., and epididymal fat pad wt. adjusted for (divided by) body wt. was made for the F2 population (SHR/NCrj × SHR/Izm) by determining the genotypes for 11 markers located within this segment. Significant linkage was observed between the rat chromosome 1 region (*D1Rat240–D1Wox28*) and fat pad wt. adjusted for body wt. with a maximum logarithm of odds (LOD) score of 4.4 at the point downstream of *D1Wox32* by 17.3 cM. Note that this linkage was independent of body wt.

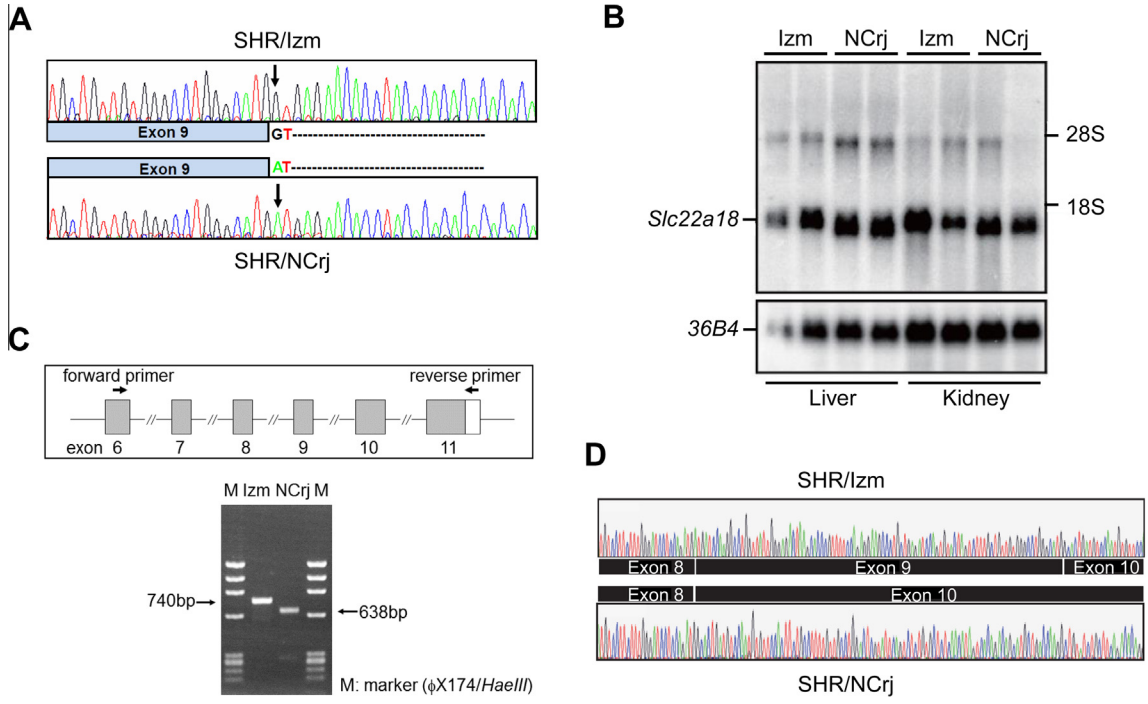

Fig. 2. Identification of a donor splice site mutation in the rat *Slc22a18* gene that results in exon skipping. (A) Comparative DNA sequencing of genomic DNA revealed a G-to-A point mutation in the donor splice site of intron 9 of the *Slc22a18* gene from strain SHR/NCrj with reduced adiposity. (B) Northern blot analysis of total RNA from the liver and kidney of each SHR using a probe for rat *Slc22a18* cDNA or *36B4*. (C) RT-PCR analysis of liver RNA with PCR-primers located on exons 6 and 11 produced differentially-migrating bands with agarose gel electrophoresis. (D) Comparative DNA sequencing of the RT-PCR products demonstrated a whole exon 9 deletion in the cDNA from strain SHR/NCrj, which was compatible with exon skipping because of the intron 9 donor splice site mutation.

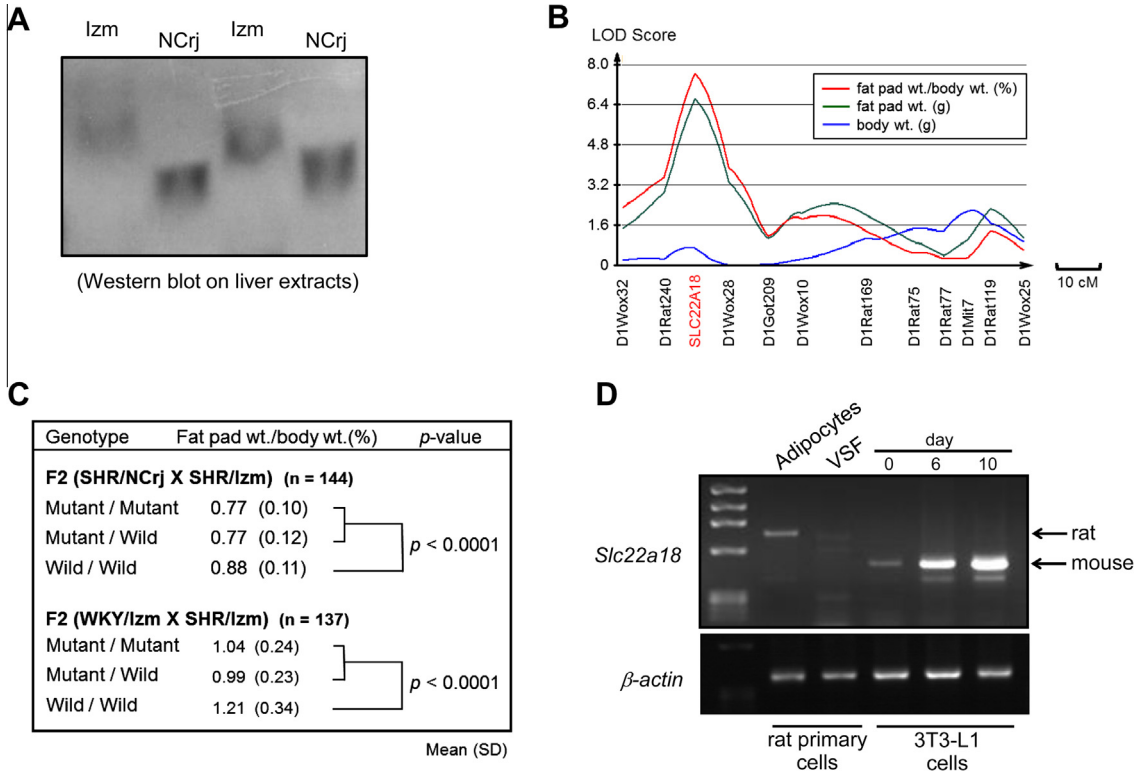

Fig. 3. Confirmation of the deletion at the protein level, localisation of the *Slc22a18* mutation at the linkage peak, replication of the linkage in another F2 cross, and *Slc22a18* mRNA expression relative to adipocyte differentiation. (A) Western blot analysis with a rabbit polyclonal antibody raised against a polypeptide of rat Slc22a18 showed differentially-migrating bands under non-denaturing conditions. (B) Including the mutation in the QTL analysis increased the maximum logarithm of odds (LOD) score up to 7.7 at the point where the rat *Slc22a18* gene is located. (C) Replication of significant linkage in two separate F2 crosses (SHR/NCrj × SHR/Izm and WKY/Izm × SHR/Izm). In both crosses, the mutant allele was significantly linked to reduced fat pad wt./body wt. in a quite similar dominant manner. (D) *Slc22a18* mRNA expression in differentiated adipocytes. RT-PCR results demonstrate that *Slc22a18* mRNA is expressed in isolated rat adipocytes rather than in the vascular stromal fraction (VSF). In 3T3-L1 cells, *Slc22a18* mRNA was abundantly expressed on Days 6 and 10 after stimulation with insulin + DEXA + IBMX in a differentiation-dependent manner.

3.3. Replication of linkage in another F2 cross

We examined the distribution of the *Slc22a18* mutation among the SHR-related strains. Unexpectedly, the mutant A-allele was prevalent among these rat strains including SHRSP or WKY rat, and the only exception was found in SHR/Izm sub-strains (Supplementary Fig. 1, Supplementary Table 1). Because all the other rat strains examined (e.g., Wistar/NCrj, Brown Norway, Sprague Dawley, Dahl/SS, Lewis) and all the reported sequences for other organisms always contained the wild-type allele, we postulated that this mutation may have originated from the ancestral Wistar colony from which both SHR and WKY were derived. This finding compelled us to make a comparison regarding the mutant *Slc22a18* allele in another F2 population derived from a cross between the SHR/Izm and WKY/Izm strains ($n = 137$). In this F2 comparison, a similar significant linkage was also observed in a similar dominant manner (Fig. 3C). This provided supporting evidence that the *Slc22a18* mutation itself or a mutation in a nearby gene should cause a decrease in adiposity independently of the genetic background.

3.4. Slc22a18 mRNA expression relative to adipocyte differentiation

Slc22a18 mRNA is ubiquitously expressed, although primarily in the liver and kidney [10]. We already observed considerable *Slc22a18* mRNA expression levels in rat adipose tissue by microarray analysis. Thus, we isolated rat primary adipocytes from a vascular stromal fraction (VSF) and examined *Slc22a18* mRNA expression by RT-PCR in these adipocytes and the VSF. These results showed that mRNA expression was mainly in isolated adipocytes but not in the VSF. Furthermore, in cultured 3T3-L1 cells, a model of preadipocyte to adipocyte differentiation [11], *Slc22a18* mRNA was expressed in a differentiation-dependent manner (Fig. 3D).

3.5. Knockdown and overexpression of Slc22a18 mRNA reciprocally regulates triglyceride accumulation and adipocyte differentiation in 3T3-L1 cells

To investigate the role of Slc22a18 in adipocyte differentiation, we examined the effects of knockdown of mouse *Slc22a18* mRNA on differentiation of 3T3-L1 cells. Adenoviral infection of either of the two independent shRNA constructs downregulated *Slc22a18* mRNA expression in 3T3-L1 cells up to less than 20% of the levels in control cells; the latter were infected with an adenoviral vector expressing *shLacZ* (Supplementary Fig. 2). On Day 8 after inducing differentiation, these control cells showed abundant lipid droplets (observed with Oil Red O staining) and massive protein expression for PPARγ and Slc22a18 (Fig. 4A). By contrast, a marked reduction in both lipid accumulation and PPARγ expression was observed in

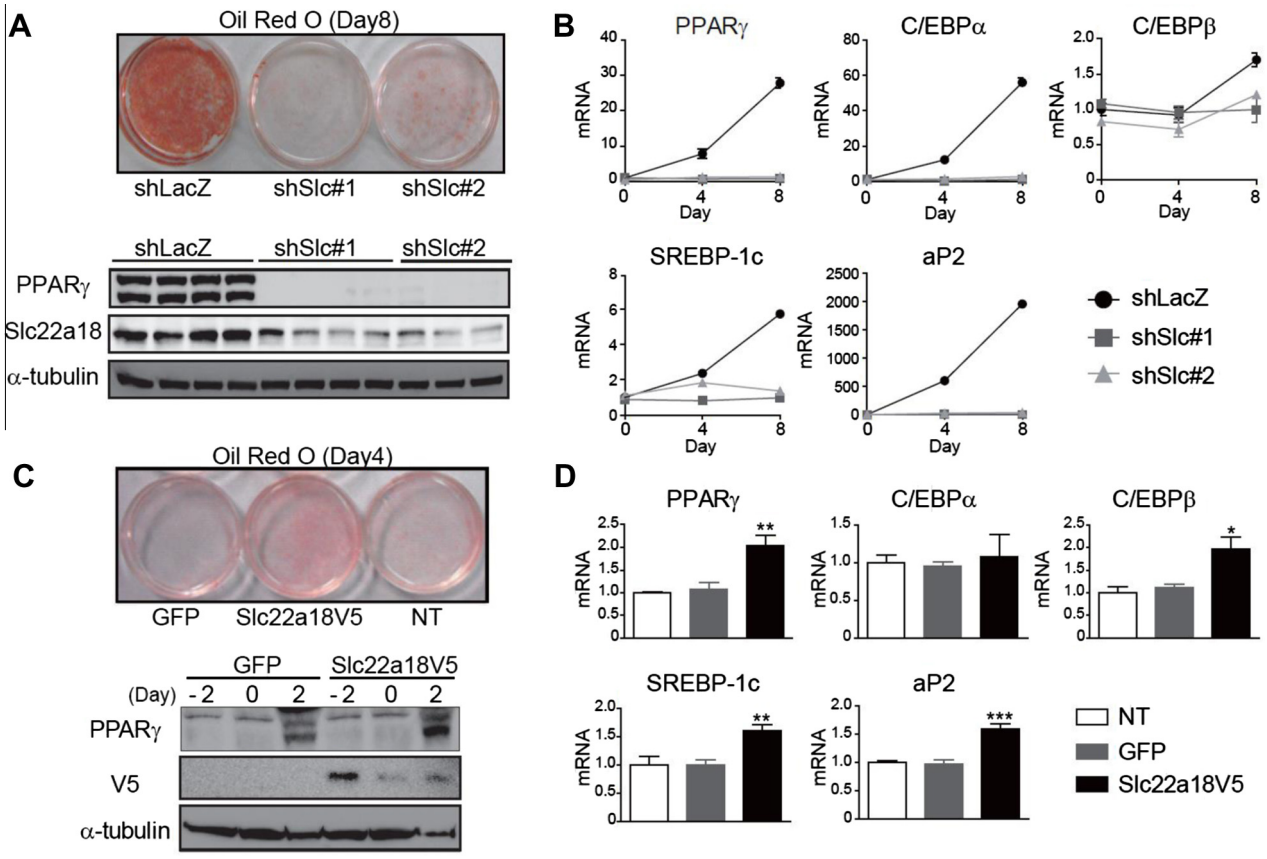

Fig. 4. The effects of knockdown and overexpression of *Slc22a18* on 3T3-L1 cell differentiation. (A) Top: triglyceride accumulation in 3T3-L1 cells on Day 8 was visualised using Oil Red O staining. Adenoviral vectors for expressing shRNA for *lacZ* or *Slc22a18* (shSlc#1 and shSlc#2) at an MOI of 30 were infected on Day −4. Bottom: western blot analysis of 3T3-L1 cells expressing each shRNA on Day 8. (B) The mRNA levels in 3T3-L1 cells expressing each shRNA on Day 0, 4, and 8 after inducing differentiation. Relative mRNA levels for adipogenic markers (PPARγ, C/EBPα, C/EBPβ, and SREBP-1c) and a mature adipocyte marker (aP2) were estimated after normalisation with 36B4. Results represent means ± SEM ($n = 3$–4 each). (C) Top: triglyceride accumulation in 3T3-L1 cells on Day 4 was detected using Oil Red O staining. The cells were infected with adenoviral vectors for expressing GFP or V5-tagged mouse Slc22a18 at an MOI of 140 or left without treatment (NT). Bottom: western blot analysis of 3T3-L1 cells expressing GFP or V5-tagged mouse Slc22a18 performed on Day −2, 0, and 2 after inducing differentiation. (D) Relative mRNA levels in each group of 3T3-L1 cells on Day 4 after inducing differentiation. Results represent means ± SEM ($n = 3$–4 each). Statistical significance in the comparison between GFP and Slc22a18V5 is shown as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the *Slc22a18*-knockdown cells. These phenotypic differences were consistent with the different mRNA expression profiles for adipocyte differentiation markers (PPARγ, C/EBPα, C/EBPβ, SREBP-1c, and aP2) whose mRNA expression levels increased progressively in the control cells but not in the *Slc22a18*-knockdown cells (Fig. 4B). On the other hand, overexpression of V5-tagged mouse Slc22a18 promoted lipid accumulation and protein expression for PPARγ (Fig. 4C) and increased mRNA expression for several adipocyte differentiation markers in mature 3T3-L1 cells on Day 4 after inducing differentiation (Fig. 4D). These results suggest that *Slc22a18* plays an important role in triglyceride accumulation and is involved in the induction of regulatory genes necessary for adipocyte differentiation.

4. Discussion

We previously reported a crucial genetic difference (*Cd36* mutation) between the SHR/NCrj and SHR/Izm strains [2]. In this study, we found a gross genome segment on rat chromosome 1 that was quite different between these SHR strains and found a significant linkage of this region to altered adiposity in the SHR. We identified a splicing mutation in the rat *Slc22a18* gene located exactly at the peak of this linkage. The mutant allele was prevalent among as well as restricted to SHR-related strains, which provides new insights into the ancestral Wistar colony from which the SHR was derived.

The SLC (solute carrier) family comprises a large number of transporters. To date, a total of 52 subfamilies and nearly 400 different genes have been identified [12]. Evidence has recently been accumulating that gene mutations in these SLC family members underlie a variety of human diseases [13,14]. However, with regard to SLC22A18 (also known as BWSCR1A, BWR1A, IMPT1, ORCTL2, and TSSC5), only a small number of papers have been published so far. Although several reports have suggested its possible association with cancer development or progression [15,16], the physiological role of SLC22A18 remains largely unknown. In this study, we found a tight linkage between *Slc22a18* mutation and reduced fat pad weight in two separate F2 comparisons. Furthermore, a significant linkage was restricted to fat pad weight or fat pad weight/body weight, but was not observed with body weight. These data seem compatible with the hypothesis that *Slc22a18* plays a role in local fat accumulation within adipose tissues. Our results demonstrating considerable *Slc22a18* mRNA expression levels in primary adipocytes and its differentiation-dependent expression, at least in 3T3-L1 cells, support the aforementioned hypothesis. More convincing evidence comes from our results demonstrating a close relationship between *Slc22a18* mRNA expression and adipocyte differentiation in cultured 3T3-L1 cells. Together, these results suggest that *Slc22a18* regulates fat accumulation *in vitro* and *in vivo*. Although further research is needed to elucidate the physiological function of Slc22a18, manipulating its function, if possible, may constitute a novel therapeutic target in obesity.

Acknowledgments

This study was supported in part by Grants-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology in Japan (to T.Y. Y.I. and T.G.) and by Grants-in-Aid for Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan (to T.G.)

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.096.

References

[1] G.M. Reaven, H. Chang, Relationship between blood pressure, plasma insulin and triglyceride concentration, and insulin action in spontaneous hypertensive and Wistar-Kyoto rats, Am. J. Hypertens. 4 (1 PT 1) (1991) 34–38.
[2] T. Gotoda, Y. Iizuka, N. Kato, J. Osuga, M.T. Bihoreau, T. Murakami, Y. Yamori, H. Shimano, S. Ishibashi, N. Yamada, Absence of Cd36 mutation in the original spontaneously hypertensive rats with insulin resistance, Nat. Genet. 22 (1999) 226–228.
[3] M. Febbraio, E. Guy, C. Coburn, F.F. Knapp Jr., A.L. Beets, N.A. Abumrad, R.L. Silverstein, The impact of overexpression and deficiency of fatty acid translocase (FAT)/CD36, Mol. Cell. Biochem. 239 (2002) 193–197.
[4] T. Gotoda, B.S. Manning, A.P. Goldstone, H. Imrie, A.L. Evans, A.D. Strosberg, P.M. McKeigue, J. Scott, T.J. Aitman, Leptin receptor gene variation and obesity: lack of association in a white British male population, Hum. Mol. Genet. 6 (1997) 869–876.
[5] N. Inoue, N. Yahagi, T. Yamamoto, M. Ishikawa, K. Watanabe, T. Matsuzaka, Y. Nakagawa, Y. Takeuchi, K. Kobayashi, A. Takahashi, H. Suzuki, A.H. Hasty, H. Toyoshima, N. Yamada, H. Shimano, Cyclin-dependent kinase inhibitor, p21WAF1/CIP1, is involved in adipocyte differentiation and hypertrophy, linking to obesity, and insulin resistance, J. Biol. Chem. 283 (2008) 21220–21229.
[6] T. Yamamoto, K. Watanabe, N. Inoue, Y. Nakagawa, N. Ishigaki, T. Matsuzaka, Y. Takeuchi, K. Kobayashi, S. Yatoh, A. Takahashi, H. Suzuki, N. Yahagi, T. Gotoda, N. Yamada, H. Shimano, Protein kinase Cbeta mediates hepatic induction of sterol-regulatory element binding protein-1c by insulin, J. Lipid Res. 51 (2010) 1859–1870.
[7] S. Wang, C.J. Basten, Z.-B. Zeng, Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC, (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) 2012.
[8] E.G. Tzortzaki, M. Yang, D. Glass, L. Deng, A.P. Evan, S.B. Bledsoe, P.J. Stambrook, A. Sahota, J.A. Tischfield, Impaired expression of an organic cation transporter, IMPT1, in a knockout mouse model for kidney stone disease, Urol. Res. 31 (2003) 257–261.
[9] H.Y. Yamada, G.J. Gorbsky, Tumor suppressor candidate TSSC5 is regulated by UbcH6 and a novel ubiquitin ligase RING105, Oncogene 25 (2006) 1330–1339.
[10] D. Dao, D. Frank, N. Qian, D. O'Keefe, R.J. Vosatka, C.P. Walsh, B. Tycko, IMPT1, an imprinted gene similar to polyspecific transporter and multi-drug resistance genes, Hum. Mol. Genet. 7 (1998) 597–608.
[11] C.S. Rubin, A. Hirsch, C. Fung, O.M. Rosen, Development of hormone receptors and hormonal responsiveness *in vitro*. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells, J. Biol. Chem. 253 (1978) 7570–7578.
[12] http://www.bioparadigms.org/slc/intro.htm.
[13] A. Enomoto, H. Kimura, A. Chairoungdua, Y. Shigeta, P. Jutabha, S.H. Cha, M. Hosoyamada, M. Takeda, T. Sekine, T. Igarashi, H. Matsuo, Y. Kikuchi, T. Oda, K. Ichida, T. Hosoya, K. Shimokata, T. Niwa, Y. Kanai, H. Endou, Molecular identification of a renal urate anion exchanger that regulates blood urate levels, Nature 417 (2002) 447–452.
[14] R. Yamada, S. Tokuhiro, X. Chang, K. Yamamoto, SLC22A4 and RUNX1: identification of RA susceptible genes, J. Mol. Med. (Berl.) 82 (2004) 558–564.
[15] C. Schwienbacher, S. Sabbioni, M. Campi, A. Veronese, G. Bernardi, A. Menegatti, I. Hatada, T. Mukai, H. Ohashi, G. Barbanti-Brodano, C.M. Croce, M. Negrini, Transcriptional map of 170-kb region at chromosome 11p15.5: identification and mutational analysis of the BWR1A gene reveals the presence of mutations in tumor samples, Proc. Natl. Acad. Sci. USA 95 (1998) 3873–3878.
[16] S.H. Chu, Y.B. Ma, D.F. Feng, H. Zhang, Z.A. Zhu, Z.Q. Li, P.C. Jiang, Correlation of low SLC22A18 expression with poor prognosis in patients with glioma, J. Clin. Neurosci. 19 (2012) 95–98.