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Citation
Scientific reports (2014), 4

Issue Date
2014-06-16

URL
http://hdl.handle.net/2433/188354

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Type
Journal Article

Textversion
publisher

Kyoto University
MicroRNA-33b knock-in mice for an intron of sterol regulatory element-binding factor 1 (Srebf1) exhibit reduced HDL-C in vivo

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MicroRNAs (miRs) are small non-protein-coding RNAs that bind to specific mRNAs and inhibit translation or promote mRNA degradation. Recent reports, including ours, indicated that miR-33a located within the intron of sterol regulatory element-binding protein (SREBP) 2 controls cholesterol homeostasis and can be a possible therapeutic target for treating atherosclerosis. Primates, but not rodents, express miR-33b from an intron of SREBF1. Therefore, humanized mice, in which a miR-33b transgene is inserted within a Srebf1 intron, are required to address its function in vivo. We successfully established miR-33b knock-in (KI) mice and found that protein levels of known miR-33a target genes, such as ABCA1, ABCG1, and SREBP-1, were reduced compared with those in wild-type mice. As a consequence, macrophages from the miR-33b KI mice had a reduced cholesterol efflux capacity via apoA-I and HDL-C. Moreover, HDL-C levels were reduced by almost 35% even in miR-33b KI hetero mice compared with the control mice. These results indicate that miR-33b may account for lower HDL-C levels in humans than those in mice and that miR-33b is possibly utilized for a feedback mechanism to regulate its host gene SREBF1. Our mice will also aid in elucidating the roles of miR-33a/b in different genetic disease models.

SREBP-1 and SREBP-2 are the major regulators of cholesterol metabolism, although there is some functional overlap among the three SREBP isoforms. Mammals possess two SREBP genes, SREBP-1 and SREBP-2 (known as SREBF1 and SREBF2, respectively) that express three major SREBP proteins. Two SREBP-1 isoforms, SREBP-1a and SREBP-1c, primarily regulate fatty acid metabolism, and SREBP-2 is the main regulator of cholesterol metabolism, although there is some functional overlap among the three SREBP isoforms.

MicroRNAs (miRs) are small non-protein-coding RNAs that bind to specific mRNAs and inhibit translation or promote mRNA degradation. Recent advances in the understanding of miR biology revealed that the genetic loci encoding for the transcription factors SREBP-1 and SREBP-2 also encode for the miRs miR-33a and miR-33b, respectively. Recent reports, including ours, indicated that miR-33a controls ABCA1 expression and reduces HDL-C levels and that miR-33a deficiency ameliorates atherosclerosis in mice. However, in rodents, a part of miR-33b is lacking from a Srebf1 intron. It is impossible to determine the precise coordinate mechanisms of miR-33a and miR-33b; the expression of these miRs is expected to depend on their corresponding host genes. Of note, SREBP-1 and SREBP-2 are differentially regulated by hormones, dietary...
challenges, or statin treatment, and the amounts and functions of miR-33a and miR-33b would be greatly affected under these conditions.

miR-33a and miR-33b are identical in their seed sequences, and thus have been predicted to repress the same set of genes with similar specificities. Antisense oligonucleotides against miR-33a are believed to simultaneously target miR-33a and miR-33b. However, there remains a 2-nucleotide mismatch after the seed sequence between miR-33a and miR-33b (Supplementary Fig. 1a), and whether this difference results in differential targeting remains to be established. Moreover, some of the previously established miR-33a target genes were not dysregulated in our miR-33a-deficient mice. Therefore, humanized mice, in which a miR-33b transgene is inserted within a Srebf1 intron, are required to address its function in vivo.

We successfully established miR-33b knock-in (KI) mice for the same intron as in humans. The protein levels of known miR-33a target genes, such as ABCA1, ABCG1, and SREBP-1, were reduced under basal conditions. An LXR agonist, which induces Srebf1 expression, enhanced miR-33b production. In vitro experiments indicated that macrophages from the miR-33b KI mice had a reduced cholesterol efflux capacity via apoA-1 and HDL-C. Moreover, HDL-C levels were reduced by almost 35% even in miR-33b KI hetero mice compared with the control mice.

The feasibility of genetic manipulation is one of the many advantages of using mice as a model organism. However, the lack of miR-33b expression was not affected by LXR stimulation. Srebf1 expression and ABCA1, SREBP-1, CPT1a, and AMPKα protein levels in the liver (Fig. 4a and Supplementary Fig. 4a). Srebf1 and miR-33b expression levels were similar in different disease models. These mice might also be useful for screening of the drugs that alter the levels of miR-33a and miR-33b.

**Results**

**miR-33b is co-expressed with SREBF1 in the human cell line HepG2.**

It is assumed that a miR located within an intron of a gene is expressed along with its host gene and exerts its specific function. Because miR-33b is located in a SREBF1 intron in humans (Supplementary Fig. 1a), we stimulated human cell line HepG2 with the LXR agonist T0901317 and determined miR-33b and miR-33a expression along with the expression of the host genes SREBF1 and SREBF2. As shown in Fig. 1a and b, miR-33b expression seemed to tag along behind SREBF1 expression. In contrast, miR-33a and SREBF2 expression was not affected by LXR stimulation (Fig. 1c and d).

**Generation of miR-33b KI mice.** Because miR-33b is located in SREBF1 intron 16 in humans and there are high homologies in exons 16 and 17 between human and mouse (82.6% of nucleotides and 79.7% of amino acids, Supplementary Fig. S1b), we introduced the human miR-33b sequence into intron 16 of mouse Srebf1. We isolated and amplified the region that encoded for the complete premiR sequence of human miR-33b and adjacent sequence, which enabled the introduction of miR-33b into intron 16 of mouse Srebf1 (Fig. 2a). Supplementary Figure S2a and Figure 2b show the results of Southern blotting analysis of genomic DNA from ES cells and tail genomic DNA from F1 mice that were successfully targeted by a KI vector, respectively. PCR analysis indicated the specific patterns for wild-type (WT), KI+/−, and KI−/− mice (Fig. 2c). This miR-33b KI strategy did not alter Srebf1 intron 16 splicing, as confirmed by PCR (Fig. 2d) and sequencing (Fig. 2e). The expression levels of miR-33b in miR-33b KI−/− mice were almost half of those in miR-33b KI+/− mice (Fig. 2f). We also measured the levels of miR-33b, miR-33a, Srebf1, and Srebf2 in WT and KI mice in both the liver and the peritoneal macrophages (Supplementary Figure S2b–d and S3a–d). Srebf1 levels were similar among these mice (Supplementary Figure S2c and S3c). Although there was no difference in miR-33a levels in macrophages (Supplementary Figure S3b), miR-33a levels were increased in proportion of the expression levels of miR-33b in the liver (Supplementary Figure S2b). The miR-33b KI−/− mice were born with the expected Mendelian ratios, were viable, fertile, and did not exhibit any obvious abnormalities in size, shape, or structure up to 8 weeks of age. Relative tissue expression pattern of miR-33b was similar to that of Srebf1 (Supplementary Fig. S2e and S2f).

**miR-33b is upregulated after inducing Srebf1 expression.** We next sought to confirm whether miR-33b expression was affected by endogenous changes in Srebf1 expression by the LXR agonist T0901317. When primary hepatocytes from the miR-33b KI−/− mice were stimulated with T0901317, Srebf1 and miR-33b mRNA levels were significantly increased in parallel, although this increase was faster for Srebf1 than for miR-33b (Fig. 3a and b). To check this effect in vivo, T0901317 was suspended in 0.5% carboxymethyl-cellulose and administrated to 8-week-old male miR-33b KI−/− mice at a dose of 25 mg/kg for 3 days. The average liver weight of the T0901317-treated mice was 1.5-fold greater than that of the control mice (Supplementary Fig. S4a). Srebf1 and miR-33b expression levels in the liver were also significantly increased in parallel (Fig. 3c and d). The average liver weight and Srebf1 expression level in the liver of T0901317-treated WT mice were parallel (Fig. 3c and d). The average liver weight and Srebf1 expression level in the liver of T0901317-treated WT mice were parallel.
Figure 2 | Generation of miR-33b knock-in (KI) mice. (a). Strategy used to generate miR-33b KI mice. (b). Southern blotting of mouse tail genomic DNA. Representative images are shown. (c). PCR analysis of mouse tail genomic DNA. Representative images are shown. (d). RT-PCR analysis of Srebf1 expression in the livers of 8-week-old mice (n = 6). N.D., not determined. Values are mean ± s.e.m.

Figure S5). As shown in Fig. 4a, Supplementary Figure S5a, and S5b, ABCA1 and SREBP-1 protein levels were lower in the livers of the miR-33b KI mice. However, the protein levels of some of the previously defined miR-33a target genes, such as CPT1a and AMPKα, remained unchanged. We also analyzed protein expressions of glucose metabolic genes (Fig. 4b and Supplementary

Figure 3 | miR-33b is co-expressed with Srebf1 in miR-33b KI mice. (a). Relative Srebf1 expression levels in primary hepatocytes from miR-33b KI mice treated with T0901317 (10 μM) for the indicated time. Values are mean ± s.e.m (n = 6). **p < 0.01 compared with the vehicle.

(b). Relative miR-33b expression levels in primary hepatocytes from miR-33b KI mice treated with T0901317 (10 μM) for the indicated time. Values are mean ± s.e.m (n = 6). **p < 0.01 compared with the vehicle. (c). Relative Srebf1 expression levels in the livers of 8-week-old male miR-33b KI mice treated with T0901317 (25 mg/kg) for 3 days. Values are mean ± s.e.m (n = 6). **p < 0.01 compared with the vehicle.
Figure 4 | miR-33b regulates ABCA1 and SREBP-1. (a). Western blotting analysis for ABCA1, SREBP-1, CPT1a, and AMPKα protein levels in the livers of WT, KI+/+, and KI+/- mice. Representative images are shown. TF2B and β-actin were used as loading controls. (b). Western blotting analysis for SRC1, PCK1, CREB and G6Pase protein levels in the livers of WT, KI+/-, and KI+/+ mice. Representative images are shown. β-actin were used as loading controls.

Figure S5c–f). However, no significant change in protein level was observed in PCK1, G6Pase, and CREB in the liver of miR-33b KI mice compared with that of control mice. SRC1 was up-regulated in miR-33b KI mice and it was opposite to the results of previous report14.

miR-33b KI reduces cholesterol efflux in macrophages. To investigate a physiological role of miR-33b in mice, we first compared the functions of peritoneal macrophages from the WT and miR-33b KI+/- mice. ABCA1 and ABCG1 protein levels were lower in macrophages from the miR-33b KI+/- mice than from the WT mice (Fig. 5a and Supplementary Figure S3e and S3f), which was compatible with the findings for our miR-33a-deficient mice. We determined apoA-I- and HDL-C-mediated cholesterol efflux from peritoneal macrophages and found that macrophages from the miR-33b KI+/- mice had lower apoA-I- and HDL-C-mediated cholesterol efflux than those from the WT mice (Fig. 5b).

A single miR-33b copy reduces serum HDL levels. Hepatic ABCA1 overexpression increases HDL-C levels, and liver-specific deletion of ABCA1 results in a substantial decrease in plasma HDL-C levels (approximately 80%) in chow-fed mice. Moreover, we previously reported that the miR-33a KI+/- mice had 22%–39% higher serum HDL-C levels than the WT mice. Thus, we determined the serum HDL-C levels of the WT, miR-33b KI+/-, and miR-33b KI+/- mice at the age of 8 weeks.

Serum HDL-C levels were significantly decreased in the miR-33b KI+/- and miR-33b KI+/- mice compared with the WT mice (Table 1). We also classified and quantified serum lipoproteins using high-performance liquid chromatography (HPLC). Mean plots of the HPLC elution profile of serum from male mice are shown in Fig. 5c, and the lipid profiles are summarized in Supplementary Table S1. These results show that only one copy of miR-33b was sufficient to substantially reduce HDL-C and total cholesterol to the same levels as in the miR-33b KI+/- mice. Moreover, the decreased HDL levels mainly comprised very large-, large-, and medium-sized HDLs (mature HDLs) (Fig. 5c and Supplementary Table S1).

Discussion

In the present study, we successfully established humanized mice, in which a miR-33b transgene was inserted within the same intron as that in human SREBF1. The LXR agonist T0901317, which is a well-established Srebf1 expression inducer, enhanced miR-33b production. The protein levels of known miR-33a/b target genes, such as ABCA1, ABCG1, and SREBP-1, were reduced under basal conditions. In vitro experiments indicated that macrophages from the miR-33b KI+/- mice had a reduced cholesterol efflux capacity via apoA-I and HDL-C. Finally, HDL-C levels were reduced by almost 35% even in the miR-33b KI+/- mice compared with the WT mice without any changes in triglyceride (TG) levels.

In contrast to humans and other mammals, rodents lack miR-33b and only have miR-33a in Srebf2. This needs to be kept in mind when attempting to directly translate to humans the previous results that miR-33a inhibition could prevent atherosclerosis in mouse models because of two reasons. First, SREBF1 and SREBF2 are differentially regulated by hormones, dietary challenges, and lipid-lowering agents, including statins. This indicates that both isoforms of miR-33 participate in regulating the primary risk factors of metabolic syndrome, which accelerate atherosclerosis. Second, miR-33b differs from miR-33a by 2-nucleotides and may have a different target profile, including stronger effects on targets in the SREBP-1-dependent regulation of fatty acid/TG homeostasis and insulin signaling. We found increased miR-33b expression after treatment with LXR agonist in our mice, which indicated that miR-33b was co-expressed with its Srebf1 host gene and enabled us to study the impact of Srebf1-derived miR-33b on cholesterol/lipid homeostasis.

We have not yet succeeded in identifying miR-33b-specific target genes. Even previously reported miR-33b target genes were not reduced in the liver of miR-33b KI mice compared with that of control mice. One of the reasons of such result may be that the previous study was conducted in human cell line and potential binding sites of miR-33b are not conserved at least in PCK1 3′UTR of mice. It is also possible that some compensated mechanisms may have occurred in miR-33b KI mice. However, the protein levels of miR-33a target genes, such as ABCA1, ABCG1, and SREBP-1, were reduced. Moreover, the protein levels of previously defined miR-33a target genes, which were not dysregulated in miR-33a KO mice, including CPT1a and AMPKα, remained unchanged. Thus, it may be necessary to assess those conditions when Srebf1 expression is strongly affected to establish the importance of the functions of miR-33b. In any event, the numbers of miR-33b transcripts were greater than those of miR-33a transcripts, and this underscores the importance of miR-33b. Although there were no differences in the levels of miR-33a in macrophages, it is interesting that the levels of miR-33a were increased in proportion to the expression levels of miR-33b in the liver. Because Srebf1 level is higher in the liver than that in macrophages, it is possible that miR-33b and miR-33a compete for the same target gene binding sites in the liver, and that the degradation of miR-33a is inhibited by miR-33b expression. In addition, there may be other unknown mechanisms.
miRs are known to target long non-coding RNAs whose functions are largely unknown, and interactions between miRs are also possible\cite{4}. Thus, miR-33b-specific functions should be determined in future experiments.

Rayner et al. recently showed that inhibiting miR-33a and miR-33b in healthy male non-human primates increased circulating HDL-C levels\cite{21}. More recently, Rottiers et al. reported that miR-33a and miR-33b acted in a redundant manner and that inhibiting both isoforms by an 8-mer LNA-modified anti-miR enhanced HDL-C levels\cite{21}. Our data demonstrated that miR-33b indeed functions to reduce cholesterol efflux and serum HDL-C levels\cite{24}. In our present miR-33b KI study and in previous miR-33a KO experiments\cite{8}, we did not observe any changes in TG levels when using miR-33a/b-targeting LNA-anti-miR treatment\cite{24}. In our present miR-33b KI study and in previous miR-33a KO experiments\cite{8}, we did not observe any changes in TG levels, indicating that modulation of miR-33s is used to increase HDL-C levels. In this context, the current LNA-modified anti-miR technique is quite potent for reducing the levels of both miR-33 isoforms and may be useful for anti-atherosclerosis therapy.

In addition to the effects on HDL-C, a study by Rayner et al. showed that miR-33 antagonism reduced very low-density lipoprotein-associated TGs in their cohort of normal male African green monkeys\cite{21}. However, Rottiers et al. did not find any significant changes in TG levels when using miR-33a/b-targeting LNA-anti-miR treatment\cite{8}. In our present miR-33b KI study and in previous miR-33a KO experiments\cite{8}, we did not observe any changes in TG levels, indicating that modulation of miR-33s is unlikely to have a strong effect on TG levels, although species differences and different dietary conditions need to be considered.

In contrast, we found a significant inhibitory effect of miR-33b on SREBP-1. A feedback system of SREBP-2 by cholesterol levels is well known, which maintains appropriate levels of cellular cholesterol.

### Table

| Serum profiling of WT, KI+/−, and KI+/+ mice |
|-----------------|-----------------|-----------------|
| **WT [n = 4]** | **KI+/− [n = 4]** | **KI+/+ [n = 4]** |
| TP (g/dl)       | 4.375 ± 0.1109  | 4.275 ± 0.04787 | 4.350 ± 0.05000 |
| ALB (g/dl)      | 2.950 ± 0.1190  | 2.825 ± 0.1109  | 2.900 ± 0.04082 |
| BUN (mg/dl)     | 21.75 ± 0.6801  | 20.58 ± 1.248   | 21.58 ± 1.680   |
| CRE (mg/dl)     | 0.1125 ± 0.00250| 0.0925 ± 0.00478| *0.0975 ± 0.006292|
| Na (mEq/l)      | 152.5 ± 0.6455  | 153.5 ± 0.2887  | 153.8 ± 0.4787  |
| K (mEq/l)       | 3.350 ± 0.05000 | 3.325 ± 0.0750  | 3.350 ± 0.1041  |
| Cl (mEq/l)      | 110.5 ± 0.6455  | 110.8 ± 0.2500  | 111.0 ± 0.5774  |
| Ca (mg/dl)      | 8.500 ± 0.1871  | 8.325 ± 0.1109  | 8.350 ± 0.06860 |
| IP (mg/dl)      | 7.775 ± 0.4589  | 7.225 ± 0.2955  | 7.400 ± 0.4637  |
| T-Bil (mg/dl)   | 0.0875 ± 0.004787| 0.0925 ± 0.008539| 0.0825 ± 0.01109|
| ALT (IU/l)      | 39.25 ± 1.702   | 33.50 ± 1.658   | 39.25 ± 1.702   |
| ALP (IU/l)      | 26.50 ± 3.663   | 21.00 ± 2.415   | 22.75 ± 1.702   |
| LDH (IU/l)      | 505.5 ± 48.55   | 398.5 ± 40.01   | 480.0 ± 29.31   |
| AMY (IU/l)      | 278.3 ± 77.21   | 243.5 ± 55.30   | 255.0 ± 55.26   |
| γ-GTP (IU/l)    | 2295 ± 68.22    | 2224 ± 62.39    | 2363 ± 97.02    |
| T-CHO (mg/dl)   | 98.50 ± 5.694   | 66.25 ± 2.287   | **62.00 ± 1.225*** |
| TG (mg/dl)      | 34.75 ± 2.780   | 32.25 ± 3.065   | 35.25 ± 4.328   |
| NEFA (μEq/l)    | 471.0 ± 47.36   | 474.8 ± 71.81   | 459.5 ± 55.01   |
| LDL-C (mg/dl)   | 6.750 ± 0.6292  | 6.750 ± 0.6292  | 6.000 ± 0.0     |
| HDL-C (mg/dl)   | 57.75 ± 4.171   | 39.25 ± 0.7500  | **37.25 ± 0.6292** |
| GLU (mg/dl)     | 216.3 ± 22.98   | 180.5 ± 8.930   | 197.8 ± 11.92   |

Values are mean ± s.e.m. Blood was obtained from chow-fed 8-wk-old male mice after 4 h fasting.

*p < 0.05;
**p < 0.01;
***p < 0.001 compared with WT mice.

Figure 5 | miR-33b reduces cellular cholesterol efflux and serum HDL-C levels. (a). Western blotting for ABCA1 and ABCG1 proteins in peritoneal macrophages from WT and KI+/+ mice. Representative images are shown. β-actin was used as the loading control. (b). Cholesterol efflux to apoA-I and HDL-C in peritoneal macrophages from WT and KI+/+ mice (n = 6 each). Values are mean ± s.e.m. **p < 0.001 compared with WT mice.

**p < 0.05;
***p < 0.01;
****p < 0.001 compared with WT mice.
However, a similar mechanism has not been established for SREBP-1c. Chronic activation of SREBP-1c in cases of overnutrition can lead to serious obesity-related problems. miR-33b may be utilized for a therapeutic modulation of miR-33 to cure metabolic diseases. Careful observations of miR-33 KI and miR-33a-deficient mice and intercrossing of these mice will enable us to detect miR-33a and miR-33b-specific target genes and to elucidate the overall functions of miR-33a and miR-33b in vivo. Moreover, our mice will aid in analyzing the roles of miR-33a/b in different genetic disease models and in screening drug candidates that can modulate miR-33a and miR-33b levels and activities.

**Methods**

**Materials.** The following antibodies were used: anti-ABCAL1 (NB400-105) and anti-ABCG1 (NB400-132) (Novus Biologicals, Littleton, CO, USA); anti-Ampara1 (#2532) and anti-Creb (#9917) (Cell Signaling Technology, Beverly, MA, USA); anti-Ctb (ab28648) and anti-Srebp1c (ab70358) (Abcam, Cambridge, UK); anti-β-actin (AC-15; A5441, Sigma-Aldrich, St. Louis, MO, USA); anti-SREBP-1 (sc-13551, sc-9894), anti-Sr1ci (sc-8995), anti-G6-Pase (sc-27198), and anti-Tf2b (sc-225) (Santa Cruz, Biotechnology, California, USA); Anti-mouse, anti-rabbit and anti-goat IgG HRP-linked antibodies were purchased from GE Healthcare (Amersham, UK). Human apoA-1 was purchased from Sigma-Aldrich. Human acetylated LDL (acLDL) and human HDL-C were purchased from Biomedical Technologies, Inc. (Stoughton, MA, USA). [1, 2-H (N)]-Cholesterol was purchased from Perkin Elmer (Boston, MA, USA). T0901317 was purchased from Cayman Chemical (Ann Arbor, MI, USA).

**Generation of miR-33b KI mice.** A targeting vector was constructed by modifying bacterial artificial chromosome RP24-310C22 (Invitrogen) using a defective prophage λ-Red recombination system. As a selection marker, a neomycin resistance cassette flanked by loxP sites (loxP-PGK-gb2-neo-loxP cassette; Gene Bridges, Germany) was inserted at the adjacent site of the human pre-miR-33b site. The targeting vector was electroporated into C57BL/6 mouse ES cells (DS Pharma Biomedical) using a Nucleofector system (Lonza). Positive clones were selected by incubating cells with 200 μg/ml geneticin (Invitrogen) for 5 days, and homologous recombination was confirmed by Southern blotting. Successfully recombined ES cells were injected into blastocysts from ICR strain mice supplied by Unitech Inc. (Japan), and chimeric mice were bred with C57BL/6 mice to generate F1 mice. F1 mice genotyping were conducted by Southern blotting. The neomycin resistance cassette was removed from the mouse germ line by breeding heterozygous mice with Ayu-1 Cre KI mice, which expressed Cre recombinase in multiple tissues, including the germ line. Descendant miR-33b knock-in heterozygous mice without the Ayu-1 Cre allele were crossed with each other to generate the miR-33b KI KI mice. All experiments were performed with male C57BL/6 background mice and wild-type littermates were used as a control. All of the experimental protocols were approved by the Ethics Committee for Animal Experiments of Kyoto University and the methods were performed in accordance with the guidelines approved by the ethics committee. Primers used for genotyping were as follows: WT/KI sense, ATGGATTAGC- TCGGTGTTAAGCACG; WT antisense, CATCCGACTAGGACCTGCTC; KI antisense, AAGTGATCCGACATGTGG; Cre sense, GTCGTCAACGGC-CGGAGCAGCT; GAGAGGAGGACCCAGGAC- ACTB; Srebf2 antisense, TAGAGCAGTCACCAGCTCAGAGG; Srebf1 antisense, GCTGCCACGACdC; and Srebf1 sense, GGGGTGTTGAAGGTCTCAAA. The membrane was blocked using 1X phosphate-buffered saline (PBS) containing 5% non-fat milk for 1 h and incubated with a primary antibody (anti-ABCAL1 (1:1000), anti-ABCG1 (1:1000), anti-Ampara1 (1:1000), anti-Ctb (1:250), anti-Tf2b (1:1000), anti-β-actin (1:3000), anti-Creb (1:1000), anti-Pck1 (1:1000), anti-Sr1ci (1:200), anti-G6-Pase (1:200) or anti-Ctb (1:1000) overnight at 4°C. After washing with PBS-0.05% Tween 20 (0.05% T-PBS), the membrane was incubated with a secondary antibody (anti-rabbit, anti-mouse and anti-goat IgG HRP-linked; 1: 2000) for 1 h at 4°C. The membrane was then washed with 0.05% T-PBS and detected with an ECL Western Blotting Detection Reagent (GE Healthcare) using an LAS-1000 system (Fuji Film).

**RNA extraction and quantitative RT-PCR (qRT-PCR).** Total RNA was isolated from tissues using TRIzol (Life Technologies, Lone). Total RNA was isolated using the TriPure Isolation Kit (Roche). cDNA was synthesized from 1 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer’s instructions. For qRT-PCR, specific genes were amplified in 40 cycles using SYBR™ Green PCR Master Mix (Applied Biosystems). Expression was normalized to that of the housekeeping gene β-actin. Gene-specific primers are as follows:

- **Srebf1** sense, AAACAAGTCCTACCGTCGAT; **Srebf1** antisense, GTTGCGAAGGGAATTGAT; **Srebf2** sense, AGGAGAACACTGTCAGTA; **Srebf2** antisense, TAAAGGAGGAGCAGCAGGA; **Actb** sense, AGGACCTCTTCAGCTCCTC; **Actb** antisense, ACTTCACTGTTGAAGCTTCTC; **Srebf1** sense, GTATGTATTCGCGATCATCAGC; **Srebf1** antisense, GTTAGTATTCGGGATCTCAG; **Srebf2** sense, GTGAGGACAAGCTTCCACACACTC; **Srebf2** antisense, GAGGGTGTTGAAGCAGCTCAGAA.

**Quantitative PCR for miRs.** Total RNA was isolated using the TriPure Isolation Kit (Roche). miR-33a and miR-33b were measured using TaqMan MicroRNA assay protocols (Applied Biosystems). Products were analyzed using a thermal cycler (ABI Prism® 7900HT sequence detection system). miR expression of samples was normalized to miR-16 using TaqMan® Endogenous Control (Applied Biosystems).

**Biochemical analysis.** Serum biochemical analysis was performed using the Hitachi 7180 Auto Analyzer (Nagahama Life Science Laboratory, Nagahama, Japan). Lipoproteins were analyzed by HPLC at Skylight Biotech (Akita, Japan), according to the procedures described previously. The percentage of radioactivity released from cells in medium relative to the total radioactivity in cells plus medium was used to determine the percentage of radioactivity released from cells. All experiments were performed in 24-well microplates at a density of 5 × 10^4 cells/ml. After incubation for 24 h, the cells were used for experiments.

**Cholesterol efflux from macrophages.** Cellular cholesterol efflux via apoA-1 was determined as described previously. In brief, thioglycollate-elicited mouse peritoneal macrophages were plated in 24-well microplates at a density of 5 × 10^5 cells/ml. Cells were cultured for 24 h in RPMI 1640 containing 10% horse serum, and then washed and incubated with RPMI 1640 with or without apoA-1 (10 μg/ml) or HDL (100 μg/ml). Cholesterol efflux was expressed as the percentage of radioactivity released from cells in medium relative to the total radioactivity in cells plus medium.

**Western blotting.** Western blotting was performed using standard procedures as described previously. A lysis buffer was supplemented with a complete mini protease inhibitor (Roche), ALN (25 μg/ml), 0.5 mM NaF, and 10 μM Na3VO4 just prior to use. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Bio-Rad). All samples (20 μg of protein) were suspended in lysis buffer, fractionated using NuPAGE 4%-12% Bis-Tris (Invitrogen) gels, and transferred to a Protran nitrocellulose transfer membrane (Whatman). The membrane was blocked using 1X phosphate-buffered saline (PBS) containing 5% non-fat milk for 1 h and incubated with a primary antibody (anti-ABCAL1 (1:1000), anti-ABCG1 (1:1000), anti-Ampara1 (1:1000), anti-Ctb (1:250), anti-Tf2b (1:1000), anti-β-actin (1:3000), anti-Creb (1:1000), anti-Pck1 (1:1000), anti-Sr1ci (1:200), anti-G6-Pase (1:200) or anti-Ctb (1:1000) overnight at 4°C. After washing with PBS-0.05% Tween 20 (0.05% T-PBS), the membrane was incubated with a secondary antibody (anti-rabbit, anti-mouse and anti-goat IgG HRP-linked; 1: 2000) for 1 h at 4°C. The membrane was then washed with 0.05% T-PBS and detected with an ECL Western Blotting Detection Reagent (GE Healthcare) using an LAS-1000 system (Fuji Film).
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