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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 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-33b and miR-33a, respectively. Recent reports, including ours, indicated that miR-33a controls ABCA1 expression and reduces HDL-C levels6–8 and that miR-33a deficiency ameliorates atherosclerosis in mice9–11. However, in rodents, a part of miR-33b is lacking from a Srebf1 intron (Supplementary Fig. 1a), and it is impossible to determine the precise coordinate mechanisms of miR-33a and miR-33b; the expression of these miR-33s is expected to depend on their corresponding host genes. Of note, SREBP-1 and SREBP-2 are differentially regulated by hormones, dietary...
In vitro experiments indicated that 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.

The feasibility of genetic manipulation is one of the many advantages of using mice as a model organism. However, the lack of miR-33a and miR-33b target genes, such as ABCA1, ABCG1, and SREBP-1, were reduced under basal conditions. An LXR agonist, which induces SREBF1 expression along with the expression of the host genes SREBF2 and SREBP-1 protein levels in the liver (Fig. 4a and Supplementary Fig. S4a). Srebf1 expression was not affected by LXR stimulation (Fig. 4a).

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. S1a), 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).

miR-33b KI results in alterations in miR-33a target proteins ABCA1 and SREBP-1. We determined ABCA1, SREBP-1, CPT1a, and AMPKα protein levels in the liver (Fig. 4a and Supplementary Fig. S2a and S2c). miR-33b is co-expressed with SREBF1 in HepG2 cells. HepG2 cells were treated with T0901317 (10 μM) for the indicated time. The relative expressions of SREBF1 (a), miR-33b (b), SREBF2 (c), and miR-33a (d) are shown (n = 6–9). Values are mean ± s.e.m. ∗p < 0.05, ***p < 0.001 compared with 0 h.
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. Sense primer was designed for exon 13, and antisense primer was designed for exon17. Note that there was no other band except for that of the correct size. Representative images are shown. (e). Sequencing alignment at the joint between exons 16 and 17 of Srebf1 in the indicated mice. (f). Relative expression of miR-33b in the livers of 8-week-old mice. Sense primer was designed for exon 13, and antisense primer was designed for exon17. Note that there was no other band except for that of the correct size. Representative images are shown. (g). Sequencing alignment at the joint between exons 16 and 17 of Srebf1 in the indicated mice. (f). Relative expression of miR-33b in the livers of 8-week-old mice. Sense primer was designed for exon 13, and antisense primer was designed for exon17. Note that there was no other band except for that of the correct size. Representative images are shown. (h). Relative expression of miR-33b in the livers of 8-week-old mice. Sense primer was designed for exon 13, and antisense primer was designed for exon17. Note that there was no other band except for that of the correct size. Representative images are shown.

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, ***p < 0.001 by one-way analysis of variance. (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 by one-way analysis of variance. (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 means ± s.e.m (n = 6). ***p < 0.001 by one-way analysis of variance. (d). Relative miR-33b 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 means ± s.e.m (n = 6). **p < 0.01 compared with the vehicle.
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 KO 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 KO 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 KO 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 KO 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\(^\text{23}\). 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\(^\text{24}\). 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\(^\text{25}\). Our data demonstrated that miR-33b indeed functions to considerably reduce miR-33b levels if pharmacological targeting 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\(^\text{26}\). However, Rottiers et al. did not find any significant changes in TG levels when using miR-33a/b-targeting LNA-anti-miR treatment\(^\text{27}\). In our present miR-33b KI study and in previous miR-33a KO experiments\(^\text{8}\), 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

<table>
<thead>
<tr>
<th></th>
<th>WT (n = 4)</th>
<th>KI(^{+/−}) (n = 4)</th>
<th>KI(^{+/+}) (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP (g/dL)</td>
<td>4.375 ± 0.1109</td>
<td>4.275 ± 0.04787</td>
<td>4.350 ± 0.05000</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>2.950 ± 0.1190</td>
<td>2.825 ± 0.1109</td>
<td>2.900 ± 0.04082</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>21.75 ± 0.6801</td>
<td>20.58 ± 1.248</td>
<td>21.58 ± 1.680</td>
</tr>
<tr>
<td>CRE (mg/dL)</td>
<td>0.1125 ± 0.002500</td>
<td>0.0925 ± 0.004787</td>
<td>* 0.0975 ± 0.006292</td>
</tr>
<tr>
<td>Na (mEq/l)</td>
<td>152.5 ± 0.6455</td>
<td>153.5 ± 0.2887</td>
<td>153.8 ± 0.4787</td>
</tr>
<tr>
<td>K (mEq/l)</td>
<td>3.350 ± 0.05000</td>
<td>3.325 ± 0.0750</td>
<td>3.350 ± 0.1041</td>
</tr>
<tr>
<td>Cl (mEq/l)</td>
<td>110.5 ± 0.6455</td>
<td>110.8 ± 0.2500</td>
<td>111.0 ± 0.5774</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>8.500 ± 0.1871</td>
<td>8.325 ± 0.1109</td>
<td>8.350 ± 0.08660</td>
</tr>
<tr>
<td>IP (mg/dL)</td>
<td>7.775 ± 0.4589</td>
<td>7.225 ± 0.2955</td>
<td>7.400 ± 0.4637</td>
</tr>
<tr>
<td>T-Bil (mg/dL)</td>
<td>0.0875 ± 0.004787</td>
<td>0.0925 ± 0.008539</td>
<td>0.0825 ± 0.01109</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>39.25 ± 1.702</td>
<td>33.50 ± 1.658</td>
<td>39.25 ± 1.702</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>26.50 ± 3.663</td>
<td>21.00 ± 2.415</td>
<td>22.75 ± 1.702</td>
</tr>
<tr>
<td>ALP (IU/l)</td>
<td>505.5 ± 48.55</td>
<td>398.5 ± 40.01</td>
<td>480.0 ± 29.31</td>
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<tr>
<td>LDH (IU/l)</td>
<td>278.3 ± 77.21</td>
<td>243.5 ± 55.30</td>
<td>255.0 ± 55.26</td>
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<tr>
<td>AMY (IU/l)</td>
<td>2295 ± 68.22</td>
<td>2224 ± 62.39</td>
<td>2363 ± 97.02</td>
</tr>
<tr>
<td>γ-GTP (IU/l)</td>
<td>3&gt;</td>
<td>3&gt;</td>
<td>3&gt;</td>
</tr>
<tr>
<td>T-CHO (mg/dL)</td>
<td>98.50 ± 5.694</td>
<td>66.25 ± 2.287</td>
<td>62.00 ± 1.225</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>34.75 ± 2.780</td>
<td>32.25 ± 3.065</td>
<td>35.25 ± 4.328</td>
</tr>
<tr>
<td>NEFA (μEq/l)</td>
<td>471.0 ± 47.36</td>
<td>474.8 ± 71.81</td>
<td>459.5 ± 55.01</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>6.750 ± 0.6292</td>
<td>6.750 ± 0.6292</td>
<td>6.000 ± 0.0</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>57.75 ± 4.171</td>
<td>39.25 ± 0.7500</td>
<td>37.25 ± 0.6292</td>
</tr>
<tr>
<td>GLU (mg/dL)</td>
<td>216.3 ± 22.98</td>
<td>180.5 ± 8.930</td>
<td>197.8 ± 11.92</td>
</tr>
</tbody>
</table>

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.
However, a similar mechanism has not been established for SREBP-1. Chronic activation of SREBP-1c in cases of overnutrition can lead to serious obesity-related problems. miR-33b may be utilized for a therapeutic method to regulate its host gene SREBF1 because insulin induces hepatic SREBP-1c expression and promotes lipogenesis and hepatic TG synthesis (Supplementary Fig. S6).

In the present study, we demonstrated the effect of miR-33b on HDL-C levels in vivo. We assume that inhibiting both miR-33a and miR-33b will have a significant effect on HDL-C levels in clinical settings. However, it is known that one miR can have hundreds of target genes and unexpected side effects may occur due to long-term therapeutic modulation of miR-33 to cure metabolic diseases. Careful observations of miR-33b 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-ABCA1 (NB400-105) and anti-APOB1 (NB400-132) (Novus Biologicals, Littleton, CO, USA); anti-AMPKα (2532) and anti-AMPKβ (2533) (Cell Signaling Technology, Beverly, MA, USA); anti-CPT1a (ab208896) (Abcam, Cambridge, UK); anti-β-actin (AC-15; A5451, Sigma-Aldrich, St. Louis, MO, USA); anti-SREBP-1 (sc-13551) (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-I was purchased from Sigma-Aldrich. Human acetylated LDL (aLDL) and human HDL-C were purchased from Biomedical Technologies, Inc. (Stoughton, MA, USA). [1, 2-3H (N)]-Cholesterol was purchased from Perkin Elmer (Boston, MA, 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 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 confirmed 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, ATGGATTTACC-AGGAGAACATGGTG-CTGA; and Exon 16 sequence primer, AGGGCAGCTCTGTAC-9TACAC.

**Southern blotting**

Southern blotting was performed using DIG High Prime DNA Labeling and Detection Starter Kit (Roche) according to the manufacturer’s protocol. Genomic DNA samples were purified and digested with MfeI and Asel for a 5′ probe, EcoRI for a 3′ probe, and NheI for a Neo probe. Primer sequences used to amplify these probes were as follows:

- 5′ probe sense, CACGGTTGATGAAATTGGATATT; 5′ probe antisense, CTGTCAGAATCTGCTGTAAGTG; 3′ probe sense, AGTTAAAATTCTCCTCCTC; 3′ probe antisense, CAGTATGCTGTTGATGATGCTTCT; Neo probe sense, GAAACGAAGGGATGCGCATC; Neo probe antisense, GTGAGTCTTACACAGTCCAG; KI antisense, AAAGTTGATCAGGTATGTTGA; Cre sense, GTCGAGACGGCGCAGGTGACATT; and Cre antisense, GTAGTTATCGCGATCATCAGCGT.

**Quantitative PCR for miRs**

Total RNA was isolated using the TRIzol Reagent (Invitrogen) and reverse-transcribed into cDNA (Superscript First Strand Synthesis Kit, Invitrogen) 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, AACAGCTCCACCTGCTGCTTAGAT; SREBF1 antisense, TTCGTGACGAAAGCCAATGTTAG; SREBF2 sense, AGGAGAACATGGTGCTGAATT; SREBF2 antisense, TAAAAGGAAAGGACACAGGA; ACTB sense, AGGCA-CTTCITCAGGGCTCCTC; ACTB antisense, GCACGGTGTGGCGGTACAGG; Srebf1 sense, TAGAGCATTACCCCAACGGT; Srebf1 antisense, GTCAGCCGCGCAGCAAGAAG; Srebf2 sense, GTGCGAGCACTGCACTCG; Srebf2 antisense, GTTGAAGTCTTACACAGGAG; Actb sense, GATCTGGCACACACACTTCT; and Actb antisense, GGCGGTTGGTAAGCTCTAA.

**RNA extraction and quantitative RT-PCR (qRT-PCR)**

Total RNA was isolated using the TRIzol Reagent (Invitrogen). cDNA was synthesized from 1 μg of total RNA using the 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, AACAGCTCCACCTGCTGCTTAGAT; SREBF1 antisense, TTCGTGACGAAAGCCAATGTTAG; SREBF2 sense, AGGAGAACATGGTGCTGAATT; SREBF2 antisense, TAAAAGGAAAGGACACAGGA; ACTB sense, AGGCA-CTTCITCAGGGCTCCTC; ACTB antisense, GCACGGTGTGGCGGTACAGG; Srebf1 sense, TAGAGCATTACCCCAACGGT; Srebf1 antisense, GTCAGCCGCGCAGCAAGAAG; Srebf2 sense, GTGCGAGCACTGCACTCG; Srebf2 antisense, GTTGAAGTCTTACACAGGAG; Actb sense, GATCTGGCACACACACTTCT; and Actb antisense, GGCGGTTGGTAAGCTCTAA.

**Quantitative PCR for miRs**

Total RNA was isolated using the TRIzol Reagent (Invitrogen). 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 using miR-16 as an internal control.

**Biochemical analysis**

After mice were fasted for 4–6 h, blood was obtained from the inferior vena cava of an anesthetized mouse, and serum was separated by centrifugation at 4°C and stored at −80°C. Employing standard methods, biochemical measurements were made using a 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.

**Statistical analysis**

Results are given as mean ± s.e.m. Statistical comparisons were made using Student’s-t tests or one-way analysis of variance with the Bonferroni post hoc test, as appropriate. A p value of <0.05 was considered significant.

26. Horiguchi, M.
24. Rottiers, V.

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**Author contributions**


**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

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