MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo.

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Supplemental Materials and Methods

Cell culture and reagents

THP-1 cells were obtained from the American Type Cell Collection (Rockville, MD, USA). THP-1 cells were transformed into macrophages by incubation for 3 days with 100 nM PMA (Nacalai Tesque, Kyoto, Japan). An immortalized primary human hepatocyte (HuS-E/2) cell line was kindly given by Makoto Hijikata (Kyoto University). Peritoneal macrophages were obtained from the peritoneal cavity of wild-type and miR-33-deficient mice 4 days after intra-peritoneal injection of 1mL of 10 % thioglycolate. The cells obtained were washed with RPMI1640 (Nacalai Tesque), spun at 1000 rpm for 10 min, and plated at a density of 106 cells/mL. Cells were washed 1h later and incubated for 2 days, then, used for experiments. The antibodies used were a polyclonal anti-ABCA1 antibody (Novus Biologicals, Littleton, CO, USA), a polyclonal anti-ABCG1 antibody (Novus Biologicals), a polyclonal anti-SREBP-2 antibody (Cayman Chemical, Ann Arbor, MI, USA), an anti-GAPDH antibody (Cell Signaling Technology, Beverly, MA, USA), and an anti-β actin antibody (Sigma-Aldrich Co, St. Louis, MO, USA). Simvastatin was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Acetylated LDL (AcLDL) was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA). ApoA-I was from Sigma-Aldrich Co. [1, 2-3H (N)]-cholesterol was from Perkin Elmer (Boston, MA, USA).

Plasmids
Expression vectors for the negative control (miR-control) and microRNAs were generated using BLOCK-iT™ Pol II miR RNAi Expression Vector Kits in accordance with the manufacturer’s protocol (Invitrogen). The miR-control vector contains a hairpin structure just as for a regular pre-miRNA, but which is predicted not to target any known vertebrate gene (pcDNA6.2-GW/EmGFP-miR-neg control plasmid). In order to create an anti-miR-33 (decoy) vector, the luciferase 3’UTR was modified to include 3-9 tandem sequences complementary to miR-33, separated by a single nucleotide space. The sequences of all constructs were analyzed using an ABI 3100 genetic analyzer. All of these constructs were correctly inserted into a pLenti6/V5-D-TOPO vector (Invitrogen) driven by a CMV promoter to stably express genes in THP-1 and HuS-E/2 cells.

**Southern blotting**

Southern blotting was performed using DIG High Prime DNA Labeling and Detection Starter Kit II in accordance with the manufacturer’s protocol (Roche).

**Primer sequences for the Southern blotting probe and genotyping**
Primer sequences for the probe (865 bp) for Southern blotting and genotyping (WT: 385 bp, KO: 491 bp) were as follows.

Southern probe primer sense; AATGCAGTGAGCAGGTGGAGTTTG
Southern probe primer antisense; ACTGCACTTGAGTTCAGACGCTAC

WT/KO sense; GGCACTACTTCTGATCCTTC
WT antisense; CAACTACAATGCACCACAGCTG
KO antisense; TTGGGATCCAGAATTCGTGATTAA

**Western blotting**

Cell lysates were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by standard western blotting procedure.

**Quantitative PCR for microRNA**

Measurement of miR-33 was performed in accordance with the TaqMan MicroRNA Assays (Applied Biosystems) protocol and the products were analyzed using a thermal cycler (ABI Prism®7900HT sequence detection system). Values were normalized using U6 snRNA expression.
**Quantitative PCR for mRNA**

Total RNA was isolated using TRIzol® reagent (Invitrogen). cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche) and PCR was performed with a SYBR Green PCR master mix (Applied Biosystems), normalized with GAPDH. An ABI Prism® 7900HT sequence detection system was used as a thermal cycler.

Gene-specific primers were as follows:

- **ABCA1** sense (human); 5’GTCCTCTTTCCGCATTATCTGG3’
- **ABCA1** antisense (human); 5’AGTTCTGGAAGGTCTTGTTCAC3’
- **SREBP2** sense (human); 5’AGGAGAACATGGTGCTGA3’
- **SREBP2** antisense (human); 5’TAAAGGAGAGGCACAGGA3’
- **LDL-receptor** sense (human); 5’CAGATATCATCAACGAAGC3’
- **LDL-receptor** antisense (human); 5’CCTCTCACACCAGCTTCC3’
- **GAPDH** sense (human and mouse); 5’TGTCATTCAACGACCCTTTC3’
- **GAPDH** antisense (human and mouse); 5’TGTGATGTGACCCCTTGC3’
- **Srebp2** sense (mouse); 5’GTGGAGCAGTCTCAACGTCA3’
- **Srebp2** antisense (mouse); 5’TGGTAGTCTCACCCAGGAG3

**Oligonucleotide sequences used for the construction of wild-type or mutant Abca1**
and Abcg1 3'UTR luciferase reporter constructs

WT Abca1:

GAACAAACTGGATACTGTACTGACACTATTCAATGCAATGCACTTCAATGC
ACGAGAACACAATTCCATTAC

Mutant Abca1:

GAACAAACTGGATACTGTACTGACACTATTCA\textcolor{red}{TACGTTACGT}CTTCA\textcolor{red}{TACGT}
ACGAGAACACAATTCCATTAC

WT Abcg1:

CTAGTACACCCAGCTGCCTGGGGCAGCAGGGACTAACGCAACGCAATGCA
CGCAATGCAGACAGTGCTGGGGTACTTA

Mutant Abcg1:

CTAGTACACCCAGCTGCCTGGGGCAGCAGGGACTAACGCAACGCA\textcolor{red}{TACGTA}
CGCA\textcolor{red}{TACGTA}GACAGTGCTGGGGTACTTA
Supplemental Figure Legends

Fig. S1. Microscopy images of THP-1 and HuS-E/2 cells.
A. miRNAs were transduced into THP-1 cells using lentivirus vectors. The transduction efficiency, which was shown using GFP, was always over 90%. THP-1 cells were induced to differentiate into macrophages by PMA stimulation (100 nM) for 3 days.
B. miRNAs were transduced into HuS-E/2 cells, which are immortalized human primary hepatocytes. The transduction efficiency, which was shown using GFP, was always over 90%.

Fig. S2. The effect of simvastatin in THP-1 macrophages.
A. Western analysis of ABCA1 protein levels following stimulation with simvastatin for 24 h at the indicated concentrations in THP-1 macrophages. GAPDH was used as a loading control.
B. Western analysis of ABCA1 protein levels following stimulation with simvastatin (10 μM) for the indicated time periods in THP-1 macrophages. GAPDH was used as a loading control.
C. Quantitative real-time PCR analysis of LDL receptor expression levels following stimulation with simvastatin (10 μM) for the indicated time periods in THP-1 macrophages. Values are the means ± S.E. of 6 independent experiments with normalization using Gapdh expression. *p<0.05 compared with 0 h.
D. Quantitative real-time PCR analysis of Srebp2 expression levels following stimulation with simvastatin (10 μM) in THP-1 macrophages. Values are the means ± S.E. of 6 independent experiments with normalization using GAPDH expression. *p<0.05 compared with 0 h.
E. Quantitative real-time PCR analysis of miR-33 expression levels following stimulation with simvastatin (10 μM) for the time indicated in THP-1 macrophages. Values are the means ± S.E. of 6 independent experiments with normalization using U6 snRNA expression. *p<0.05 compared with 0 h.

Fig. S3. Silencing of endogenous miR-33 using a decoy gene in vitro.
A. Schema of the decoy gene. The luciferase 3’UTR was modified to include 3-9 tandem sequences complementary to miR-33 each separated by a single nucleotide spacer.
B. 293T cells were transfected with control-luc or decoy-luc (x3, x6, and x9) constructs,
along with the expression vector of miR-33. Luciferase activity was measured 48 h after transfection. The reduction in luciferase activity indicates the effect of the decoy gene. Values are the means ± S.E. of 3 independent experiments. ***p<0.001. *p<0.05.

C. Western analysis of ABCA1. THP-1 cells were transfected with control-luc or decoy (anti-miR-33 x9)-luc using a lentivirus vector. Cells were cultured in RPMI 1640 with 10% FBS, otherwise cells were cultured without FBS or treated with simvastatin (10 µM) for 24 h. GAPDH was used as a loading control.

Fig. S4. Comparison of \textit{Srebp2} expression in 8-week-old mice.
Quantitative real-time PCR analysis of \textit{Srebp2} in the liver of 8-week-old male and female mice. Values are the means ± S.E. of 3-4 mice with normalization using \textit{Gapdh} expression. The value for wild-type male mice was set at 1.0.

Fig. S5. Comparison of \textit{Srebp2} expression in 16- and 24-week-old mice.
A. RT-PCR analysis of \textit{Srebp2} in the liver of 16-week-old male (upper) and female (lower) mice. The sense primer was designed in exon 16 of \textit{Srebp2} and the antisense primer was designed in exon 17 of \textit{Srebp2}. \textit{Gapdh} expression was used as a control.
B. Quantitative real-time PCR analysis of \textit{Srebp2} in the liver of 16-week-old male and female mice. Values are the means ± S.E. of 3-4 mice with normalization using \textit{Gapdh} expression. The value for wild-type male mice was set at 1.0.
C. RT-PCR analysis of \textit{Srebp2} in the liver of 24-week-old male (upper) and female (lower) mice. The sense primer was designed in exon 16 of \textit{Srebp2} and the antisense primer was designed in exon 17 of \textit{Srebp2}. \textit{Gapdh} expression was used as a control.
D. Quantitative real-time PCR analysis of \textit{Srebp2} in the liver of 24-week-old male and female mice. Values are the means ± S.E. of 3-4 mice with normalization using \textit{Gapdh} expression. The value for wild-type male mice was set at 1.0.

Fig. S6. The expression level of miR-33 in mice.
A. Quantitative real-time PCR analysis of miR-33 and \textit{Srebp2} in 8-week-old wild-type male mice with normalization using U6 snRNA or \textit{Gapdh} expression. The values for the liver were set at 1.0.
B. Quantitative real-time PCR analysis of miR-33 in the kidney of 8-week-old male mice (N.D.: not determined).
C. Quantitative real-time PCR analysis of miR-33 in the brain of 8-week-old male mice (N.D.: not determined).
D. Quantitative real-time PCR analysis of miR-33 in the liver of 16-week-old male and female mice (N.D.: not determined)

Fig. S7 Analysis of ABCG1 *in vitro* and *in vivo*.
A. Sequence alignment of *Abcg1* 3’UTR. There are 2 potential miR-33 binding sites in the *Abcg1* 3’UTR; however, these were conserved only in rodents (not human).
B. 293T cells were transfected with wild-type or mutant *Abcg1* 3’UTR luciferase constructs, along with the expression plasmids for miR-control (negative control), miR-33, and miR-146a. Values are the means ± S.E. of 4 independent experiments. *p<0.05 compared with other columns.
C. Western analysis of hepatic ABCG1 in 16-week-old male mice. GAPDH was used as a loading control.
D. Western analysis of hepatic ABCG1 in 16-week-old female mice. GAPDH was used as a loading control.
Supplemental Figure 1
Supplemental Figure 2

A

Simvastatin 24 h

0 1 10 50 (μM)

ABCA1

GAPDH

B

Simvastatin (10 μM)

0 h 6 h 24 h

ABCA1

GAPDH

C

LDL-receptor/GAPDH

0 h 6 h 24 h

D

SREBP2/GAPDH

0 h 6 h 24 h

E

miR-33/U6

0 h 6 h 24 h

Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 4

Relative expression level vs. genotype in males and females at 8 weeks for the Srebp2 gene.
Supplemental Figure 5

A

Srebp2
(exon 16 to exon 17)

+/

-/

Gapdh

+/

-/

B

Srebp2 (16 week)

Male (+/+)

Male (-/-)

Female (+/+)

Female (-/-)

C

Srebp2
(exon 16 to exon 17)

+/

-/

Gapdh

+/

-/

D

Srebp2 (24 week)

Male (+/+)

Male (-/-)

Female (+/+)

Female (-/-)
Supplemental Figure 6

A

miR-33

Srebp2

B

miR-33 (Kidney)

C

miR-33 (Brain)

D

miR-33 (Liver)

Supplemental Figure 6
Supplemental Figure 7

A

Abcg1 3'UTR

miR-33 binding site

Mmu (Mouse)

CUGCGUCGGGCAACGGAUGACAACG

CAAUGCAACG

GACAGUGCUGGGG

Rno (Rat)

CUGCGUCGGGCAACGGAUGACAACG

CAAUGCAACG

GACAGUGCUGGGG

********** ***********

1

2

B

Abcg1 3'UTR

Relative luciferase activity

WT

Mutant

C

ABCg1

GAPDH

D

+/-

-/-

ABCg1

GAPDH

Supplemental Figure 7