The regulation of PCSK9 and cholesterol homeostasis by microRNAs

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The regulation of PCSK9 and cholesterol homeostasis by microRNAs

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Chan Joo Lee

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This certifies that the Doctoral Dissertation

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The regulation of proprotein convertase subtilisin/kexin type 9 (PCSK9) and cholesterol homeostasis by microRNAs

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Low density lipoprotein (LDL) receptor on the cellular membrane is a receptor responsible for uptake of serum LDL-cholesterol into the cell. Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to the LDL receptor (LDLR), followed by internalization of the complex and degradation of LDLR, and ultimately regulates cholesterol homeostasis. Transcription of PCSK9 is regulated by SREBP (sterol regulatory element binding protein) but

regulation by microRNA (miRNA) is still not known. The purpose of this study is to show the existence of miRNA regulating PCSK9 and involving cholesterol homeostasis. In order to discover the posttranscriptional regulation mechanism of PCSK9, bioinformatic algorithms were utilized to discover miRNAs expected to target PCSK9. Overexpression of these miRNAs reduced both *PCSK9* mRNA and protein significantly whereas increased LDLR protein. 3′UTR (untranslated region) luciferase reporter assay showed that these miRNAs downregulated PCSK9 expression by binding 3′UTR of PCSK9. Site-directed mutagenesis of predicted seed region in *PCSK9* mRNA revealed specific interaction between miRNAs and *PCSK9* mRNA. In Dil-LDL uptake assay, overexpression of these miRNA demonstrated a relative increase in Dil-LDL uptake by 70% or greater. In addition, it was indentified that miR-224 regulated the inducible degrader of the LDL receptor (IDOL) by 3′UTR reporter assay. These indicate that miRNAs may be yet another mechanism involved in cholesterol homeostasis by posttranscriptional regulation of PCSK9 or IDOL and that utilization of miRNA may be a method of treatment of hypercholesterolemia.

Key words: PCSK9, LDL receptor, microRNA, hypercholesterolemia

The regulation of proprotein convertase subtilisin/kexin type 9 (PCSK9) and cholesterol homeostasis by microRNAs

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I. INTRODUCTION

Proprotein convertase subtilisin/kexin 9 (PCSK9), a type of proteinases that undertake restrictive proteolysis, is known to promote the breakdown of low density lipoprotein receptor (LDLR), which uptakes cholesterol at the cell membrane and regulates the cholesterol level in the blood. Typically, LDLR is endocytosed when it binds to lipoproteins on the surface of the cell membrane, then recycled after the bound lipoprotein is dissociated under the low pH environment in the endosome.[1,2] PCSK9 binds to the first epidermal growth

factor-like repeat A domain of LDLR, and the PCSK9-LDLR complex is endocytosed even without binding of lipoproteins to LDLR. LDLR on the PCSK9-LDLR complex is directed to the lysosome and degraded instead of being recycled.[3] The gain of function mutation of *PCSK9* results in a reduction of LDLR, leading to an increase in blood LDL cholesterol concentration.[4] On the contrary, the loss of function of *PCSK9* results in relatively less LDLR destruction, leading to increased absorptions of LDL cholesterol into the cell, and ultimately lowers the incidence of coronary artery disease of which the main risk factor is hypercholesterolemia.[5] By altering the expression level of LDLR, the qualitative and quantitative change of PCSK9 affects cholesterol related diseases.

Cellular cholesterol is regulated by a transcriptional factor called sterol regulatory element binding protein (SREBP), especially by SREBP-2.[6] An increase in *SREBP-2* transcription due to a decrease in cellular cholesterol level leads to an increased expression of LDLR, which manages the absorption of LDL cholesterol in the blood and the expression of 3-hydroxy-3-methyglutaryl coenzyme A reductase (HMG-CoA reducatase), the most critical enzyme in synthesizing cholesterol.[7,8] Moreover, SREBP-2 causes an increase not only in LDLR expression, but also in PCSK9 expression.[9,10]

The primary mechanism by which statin-related substances, the medicine

for hypercholesterolemia, decrease the blood cholesterol level is to reduce endogenous cholesterol synthesis by blocking HMG-CoA reductase in liver cells. The compensatory mechanism that increases the uptake of LDL is mediated by increased LDLR expression by the activation of SREBP-2 driven by the low level of cellular cholesterol. However, the expression of PCSK9 also increases as the expression of LDLR increases because PCSK9 is the target of SREBP-2, hindering the statins from decreasing the plasma cholesterol level. In case when PSCK9 was knocked-out, it was observed that the expression of LDLR increases in a greater amount by statin than that in wild-type, thus the blood cholesterol level drops more effectively.[11] Therefore, it is expected that the use of PCSK9 inhibitors will augment the effect of statin, and the benefit of PCSK9 inhibitors is currently under extensive studies as a therapeutic modality of hypercholesterolemia treatment.

Recently, the role of microRNA (miRNA) on gene expression is being widely studied, particularly in the field of cancer biology. miRNAs are small non-coding RNAs which consist of ~22 nucleotides, bind to the 3'-untranslated region (UTR) of target mRNAs and regulate the post-transcriptional expression of target genes.[12] Up to now, about thousands of miRNA has been discovered and since one miRNA is able to target many different genes, it is thought about miRNA that over 30% of human genes can

be regulated by miRNA.[13] Mainly, researches of miRNA have focused on cancer, specifically related to cell activities such as tumorigenesis, differentiation, metabolism, and apoptosis have been under progress.

Researches on cell metabolism and miRNA have become more active recently and researchers like Rayner et al. have discovered that miR-33 represses the expression of adenosine triphosphate-binding cassette (ABC) transporter, which has the role of secreting cellular cholesterol out of the cell in the form of apolipoprotein A1 (ApoA1).[14] Also, it is already a widely known fact that miR-122 is involved in the metabolism of fatty acid and cholesterol.[15] Moreover, it has been discovered that miRNA-370 is involved in the oxidation process of fatty acid through regulating miR-122 expression.[15] However, researches on miRNAs particularly targeting genes that role in the cellular lipid metabolism are still at an early stage, thus it is thought that more discoveries will be found in this field.

The purpose of this research is to discover a new mechanism for regulating cellular cholesterol level mediated by miRNAs targeting PCSK9. Finding miRNAs that regulate the expression of PCSK9 will be a potential modality of hypercholesterolemia treatment.

II. MATERIALS AND METHODS

1. General methods and supplies

DNA manipulations were performed using standard molecular biology techniques. Delipidated serum (DLPS) was prepared from FBS as described by Hannah et al.[16] Protein concentrations were determined using a BCA kit (Pierce, Rockford, IL USA). Cell culture medium and reagents were obtained from invitrogen (Calsbard, CA, USA). *N*-acetyl-leucine-leucine-norleucinal was obtained from Merck Biosciences (Calbiochem, San Diego, CA, USA). Rosuvastatin was kindly provided by AstraZeneca (Mölndal, Sweden). Sodium mevalonate was prepared from mevalonic acid lactone (Sigma-Aldrich, Co. St. Louis, MO, USA) as follows.[17] To prepare 1.0 M sodium mevalonate, 5 g of mevalonic acid lactone was dissolved in distilled water, then 4 ml of 10 N NaOH was added drop-wise and stirred for 40 min at room temperature. The pH of the solution was adjusted by 0.5 N HCL to pH 7.5, then the final volume was brought to 38.4 ml with distilled water. The stock solution was filter-sterilized, divided into aliquots, and stored at -20°C until use. mirVana™ negative control (miR-NC, Cat #4464059), mirVana™ miRNA mimics (miR-224, ID: MC12571; miR-1912, ID: MC14695) and mirVana™ miRNA inhibitors (miR-224, ID: MH12571; miR-1912, ID:

MH14695) were purchased from Ambion Inc. (Bedford, MA, USA). Dil-LDL was purchased from Biomedical Technologies Inc (Stoughton, MA, USA). Other reagents not specified were obtained from Sigma-Aldrich. The following antibodies were used in the current studies: anti-IDOL from Abcam (Cambridge, UK), and anti-GAPDH from Cell signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce. The polyclonal antibodies against human PCSK9, SREBP-2 were prepared as previously described by Jeong et al.[10] The polyclonal antibody against human LDLR was raised in rabbits using synthetic peptide spanning amino acids 832-841 of bovine LDLR according the standard technique.

2. Cell culture and transfection

HepG2 (American Type Culture Collection number HB-8065) cells were maintained in medium A (DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) FBS. Transfection of miRNA mimics or miRNA inhibitors into HepG2 cells in suspension was carried out using Lipofectamine™2000 (Invitrogen) according to the method described by Notarangelo et al. with minor modifications.[18] Briefly, miRNA mimics or miRNA inhibitors were complexed in Opti-MEM (Invitrogen)

using Lipofectamine™2000 according to the manufacturer's instruction. While the miRNA mimics or inhibitor-lipofectamine complex was prepared, HepG2 cells were trypsinized and suspended in medium A supplemented with 10% FBS. The complex was mixed with 2×10^5 cells per well/12-well plate in 0.8 ml of medium A supplemented with 10% FBS and rocked gently for 20 min at 37°C in the tube. Aliquots of mixtures were plated on 12-well plates and cultured overnight at 37°C under a humidified atmosphere of 5% CO_2. For the treatment of cells with or without sterols, on day 1, cells were washed twice with PBS and changed to medium A supplemented with 10% DLPS, 0.1 μM rosuvastatin, and 50 μM sodium mevalonate in the absence or presence of 1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol added in a final concentration of 0.2% (v/v) ethanol. On day 2, cells were washed twice with PBS, harvested for further analyses. Transfection of plasmids into HEK293 cells was carried using Lipofectamine™2000 (Invitrogen) according to the manufacturer's instruction.

3. Quantification of miRNA and mRNA expression

Total RNA including the miRNA fraction was isolated using miRNeasy micro kit (Qiagen, Hiden, Gemany) according to the manufacturer's instruction. Removal of DNA from RNA was achieved with RNase-free

DNase (Qiagen). For mRNA quantification, cDNA was synthesized from 2 μg of DNase-treated RNA using a High capacity cDNA synthesis kit (Applied Biosystems, Foster sity, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed using the PowerSYBR® Green PCR master (Applied Biosystems). For miRNA quantification, cDNA was synthesized from 10 ng of total RNA using specific miRNA primers supplied by the specific TaqMan® Micro Assay and TaqMan® MicroRNA reverse transcription kit (Applied Biosystems). TaqMan® Universal master mix and specific TaqMan® Micro Assay (Applied Biosystems) were used for qRT-PCR All reactions were analyzed using the StepOne™ Real-time PCR systems (Applied Biosystems). *GAPDH* and *U6 snRNA* were used as references for mRNA and miRNA, respectively. Each sample was analyzed in triplicate and the relative amounts were quantified by the comparative cycle-time method. The primers and TaqMan® Micro Assay used for qRT-PCR are shown in Tables 1 and 2.

Table 1. Primers used for quantitative real-time PCR

Gene symbol	NCBI Accession No.	Sequence (5′ to 3′)
GABRE	NM_174936.3	FW: GGCAGGTTGGCAGCTGTTT RV: CGTGTAGGCCCCGAGTGT
GAPDH	NM_001256799.2	FW: TGTGTCCGTCG-TGGATCTGA RV: CCTGCTTCACCACCTTCTTGAT
LDLR	NM_000527.4	FW: GGCTGCGTTAATGTGACACTCT RV: CTCTAGCCATGTTGCAGACTTTGT
PCSK9	NM_174936.3	FW: GGCAGGTTGGCAGCTGTTT RV: CGTGTAGGCCCCGAGTGT
SREBF2	NM_004599.3	FW: CGGTAATGATCACGCCAACAT RV: TGGTATATCAAAGGCTGCTGGAT

Table 2. Taqman assays for quantitative real-time PCR

miRbase ID	miRBase Accession No.	TaqMan Assay ID
U6 snRNA	NR_004394	001973
hsa-miR-224	MIMAT0007887	002099
has-miR-1912	MIMAT0000281	121110_mat

4. Immunoblot analysis

After treatment of cells as described in each figure legend, cells were washed twice with PBS and lysed with 200 μl of NUN buffer containing 0.33 M NaCl, 1.1 M urea, 1% Nonidet P-40, 25 mM HEPES (pH 7.6), and protein inhibitors (1 mM DTT, 10 μg/ml leupeptin, 1 mM PMST, 2 μg/ml aprotinin, and 50 μg/ml *N*-acetyl-leucine-leucine-norleucinal) by adding directly onto the plate.[19] Cell lysates were harvested and further vortexed at room temperature for 10 min for the complete liberation of proteins. Lysates were cleared by centrifugation at 20,000 g for 15 min at 4°C, and the supernatants were collected as whole cell lysate. After quantitation of protein, aliquots of proteins were subjected to 8% SDS-PAGE and transferred onto nitrocellulose ECL membranes (GE healthcare, Piscataway, NJ, USA), and immunoblot analyses were performed using the Supersignal West Pico Chemiluminescent Substrate System (Pierce).

5. Screening of miRNAs targeting PCSK9

The 3′UTR sequence of the human *PCSK9* mRNA was retrieved using Entrez (http://www.ncbi.nlm.nih.gov/entrez/). Then the sequence was analyzed to reveal binding sites for potential miRNAs targeting human *PCSK9* by using on-line computational algorithm: miRanda

(http://www.microrna.org) and TargetScan (http://www.targetscan.org). Then, predicted miRNAs were listed according to the Context+ score or the mirSVR score by TargetScan or miRanda, respectively.

6. The luciferase reporter assay

The *PCSK9* 3′UTR luciferase reporter (pLightSwitch_PCSK9_3UTR) construct harboring the complete 3′UTR of the human *PCSK9* mRNA at down-stream of renilla luciferase gene was purchased from Switchgear Genomics (Melno Park, CA, USA). Site-directed mutagenesis of the putative seed regions in the 3′UTR for miRNAs was performed using *pfu* DNA polymerase (Agilent technologies, Palo Alto, CA, USA) to create mutant variants of the *PCSK9* 3′UTR luciferase reporter construct. Primer sequences and genomic location used for mutagenesis are shown in Table 3. For luciferase reporter assays, HEK293 cells were plated in a 24-well plate and then cotransfected with 80 ng of firefly luciferase control vector (pGL3-control, Promega, Madison, WI, USA), 200 ng of the 3′UTR luciferase reporter construct, and 40 nM (final concentration) of miRNA mimic using Lipofectamine™2000 (Invitrogen) according to the manufacture's instruction. Cells were lysed 48 h after transfection and luciferase activity was measured by Dual Luciferase Reporter Assay System (Promega). The pGL3-Control

was cotransfected with the construct as an internal control to standardize transfection efficiency. The renilla luciferase activity representing the activity of the 3′UTR of *PCSK9* mRNA was normalized to the firefly luciferase activity driven by pGL3-Control vector, and the amount of protein in the lysate. Transfection were done in duplicate and repeated at least thrice in independent experiments. The results were shown as the fold change between selected miRNA mimics and a negative control miRNA mimic for each 3′UTR vector.

Table 3. Primers used for site directed mutagenesis of *PCSK9* 3′UTR luciferase reporter

Plasmid		Sequence (5′-3′)
Mut-1	FW	ATTAATATGGTGACT***G***TTTAAAATAAAAACA
Mut-2	FW	ATTTTTATTAATATG***T***TGAC***G***TTTTAAAATAA
Mut-3	FW	TTTTTATTAATATGGTG***C***CTTTTTAAAATAAAAAC
Mut-4	FW	GAAGATATTTATTCT***T***G***T***TTTTGTAGCATTTTT
Mut-5	FW	TTGAAGATATTTATT***A***TGGGTTTTGTAGCAT
Mut-6	FW	CTTGAAGATATTTAT***AA***TGGGTTTTGTAGCATT

(miRNA target sites are underlined and point mutations are indicated in italic.)

7. Fluorescence microscopy and LDL uptake assay

Twenty four hr after transfection, cells were incubated with 10% DLPS culture medium for 24 h followed by treatment for 3 h with 2 μg/ml of fluorescence-labeled Dil-LDL. Cells were washed twice with PBS and fluorescence images were gained by using fluorescence microscope (Olympus, Tokyo, Japan) with rhodamine filter. For quantification of LDL uptake, cells were trypsinized to obtain a single-cell suspension. The mean fluorescence intensities of 10,000 cells were analyzed by fluorescence-activated cell sorting on the FACScan (BD Bioscience, San Jose, CA USA).

8. Human liver samples

Human liver specimens were obtained from Severance Hospital Gene Bank at Yonsei University College of Medicine (Seoul, Korea). This study was a retrograde study and all protocols were approved by the Institutional Review Board of Yonsei Health Systems (Seoul, Korea, IRB No. 4-2013-0011). The histological classification of the specimen as a tumor or the matched adjacent normal tissue followed the data provided by the Severance Hospital Gene Bank.

9. Statistical analysis

Three experiments were performed for all *in vitro* studies. The results are presented as means ± standard deviation (SD). The data were subjected to a two-tailed Student's *t*-test. The Spearman's rank-order correlation coefficient testing was used to evaluate the strength of a link between miRNA and protein levels. Statistical analyses were carried out using SPSS version 18.0 for Windows (Statistical Package for the Social Science, SPSS, Ins., Chicago, USA). All *P* values less than 0.05 were considered statistically significant.

III. RESULTS

1. Bioinformatic analyses of potential miRNAs regulating PCSK9

To search miRNA candidates targeting PCSK9, two algorithms including TargetScan and miRanda were used. Both algorithms predicted several miRNAs to be candidates targeting PCSK9. miRNAs with lower Context+ score in Targetscan or lower mirSVR score in miRanda might be more favorable candidates as regulator of target gene, they were listed in order of score (Table 4). Six miRNAs, miR-24, miR-99b, miR-149, miR-224, miR-601, and miR-1912, which were overlapped in top 10 ranked lists of both algorithms, were selected for further studies.

Table 4. Top 10 possible PCSK9 targeting miRNAs predicted by TargetScan and miRanda

TargetScan		miRanda	
miRNA	**Context+ score**	**miRNA**	**mirSVR score**
has-miR-99b	**-0.52**	has-miR-29b	-1.25
has-miR-24	**-0.52**	has-miR-187*	-1.23
has-miR-191	-0.38	**has-miR-1912**	**-1.11**
has-miR-1912	**-0.34**	**has-miR-224**	**-1.09**
has-miR-1972	-0.3	**has-miR-601**	**-1.05**
has-miR-601	**-0.29**	has-miR-616*	-1.04
has-miR-564	-0.29	has-miR-374b	-0.80
has-miR-224	**-0.27**	**has-miR-24**	**-0.57**
has-miR-149	**-0.24**	**has-miR-149**	**-0.44**
has-miR-571	-0.24	**hsa-miR-99b**	**-0.35**

(Overlapped miRNAs are indicated in bold.)

2. Screening of putative miRNAs regulating PCSK9 by immunoblot assay

To determine whether selected miRNAs affect PCSK9 expression in vitro, amounts of PCSK9 and LDLR were evaluated after transient transfection of each miRNA in HepG2 cell (Fig. 1). Among predicted miRNAs, miR-1912 reduced the amount of PCSK9 most effectively with marked increase in the amount of LDLR. miR-224 reduced the amount of PCSK9 slightly, however, the increase in the amount of LDLR was considerably high comparable to that increased by miR-1912. miR-149 reduced the amount of PCSK9, however, it showed no effect on LDLR. Effects of miR-24, miR-99b, and miR-601 on amounts of PCSK9 and LDLR were relatively small compared to that of miR-224 and miR-1912. Accordingly, miR-224 and miR-1912 were selected for further evaluation for its potentials of regulating PCSK9 and cellular cholesterol metabolism via LDLR.

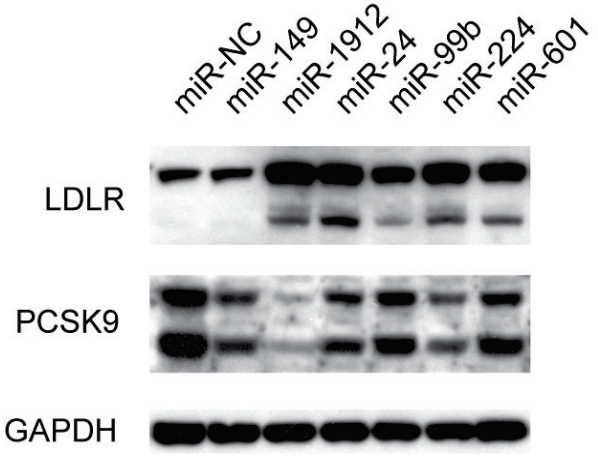


Figure 1. Effects of miRNAs which were predicted to target PCSK9 by bioinformatic algorithms on the expression of LDLR and PCSK9

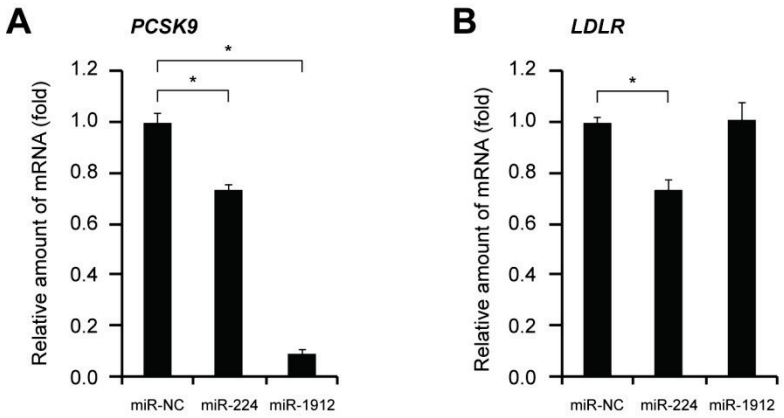
On day 0, HepG2 cells were transfected with each miRNA mimic at a final concentration of 40 nM as described in Material and Methods. On day 1, cells were switched to fresh medium A supplemented with 10% delipidated serum (DLPS). On day 2, cells were switched to fresh medium A supplemented with 10% DLPS and 0.1 μM of rosuvastatin and 0.05 mM of sodium mevalonate. On day 3, 16 h after incubation, cells were harvested and immunoblot analysis was carried out with polyclonal antibodies against PCSK9, and the low density lipoprotein receptor (LDLR). GAPDH was used as a loading control.

3. miR-224 and miR-1912 regulate amounts of PCSK9 and LDLR proteins

The change of PCSK9 and LDLR expression was validated by qRT-PCR (Fig. 2A, B). The amount of *PCSK9* mRNA was significantly decreased by miR-224 and miR-1912. miR-224 reduced the amount of *LDLR* mRNA, while miR-1912 did not. The reduction of *LDLR* mRNA could be caused either by direct action of miR-224 or secondarily by the change of cholesterol metabolism driven by miR-224-mediated changes in PCSK9 expression. Immunoblot analysis revealed that these miRNAs reduced PCSK9 protein level regardless of cholesterol treatment in the cells and effectively blocked rosuvastatin-induced up-regulation of PCSK9 (Fig. 2C). The amount of LDLR protein was increased accordingly. The amount of *SREBP-2* mRNA was decreased by both miR-224 and miR-1912. However, the protein level of the nuclear form of SREBP-2, which activates *PCSK9* transcription, was not affected by miRNAs.

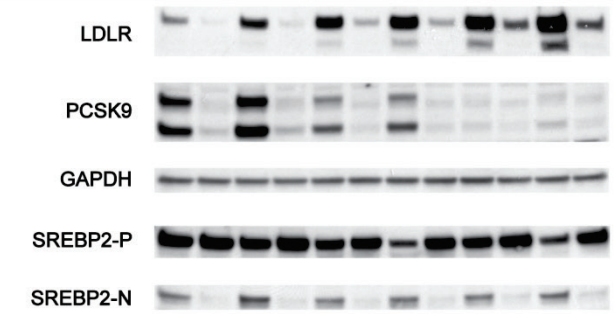
Effects of miR-1912 to reduce PCSK9 protein and to increase LDLR protein were revealed to be in a dose-dependent manner up to final concentration of 80 nM (Fig. 2E). MiR-224 caused the similar dose-dependent reduction of PCSK9 protein and increase in LDLR protein (Fig. 2D). On the contrary, when cells were cotransfected with miRNAs and antisense

oligonucleotides (anti-miR, miRNA inhibitors), which silence the action of miRNAs, the effects of miRNAs on PCSK9 and LDLR protein were attenuated significantly (Fig. 2F).



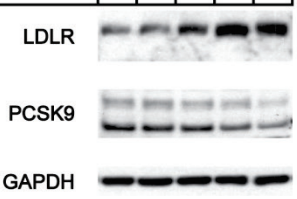
C

	miR-NC				miR-224				miR-1912			
Rosuvastatin	-	-	+	+	-	-	+	+	-	-	+	+
Sterol	-	+	-	+	-	+	-	+	-	+	-	+



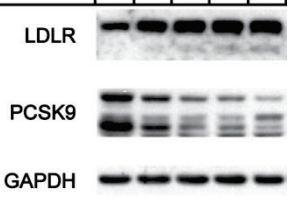
D

miR-224 (nM)	0	20	40	60	80
miR-NC (nM)	80	60	40	20	0



E

miR-1912 (nM)	0	20	40	60	80
miR-NC (nM)	80	60	40	20	0



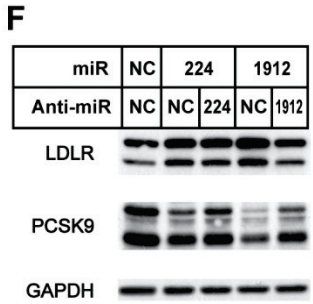


Figure 2. Effects of miR-224 and miR-1912 on the expression of LDLR and PCSK9 in HepG2 cells

(A, B) On day 0, HepG2 cells were transfected with miRNAs at a final concentration of 40 nM as described in Material and Methods. On day 1, cells were switched to fresh medium A supplemented with 10% DLPS. On day 2, cells were switched to fresh medium A supplemented with 10% DLPS, 0.1 μM of rosuvastatin, and 0.05 mM of sodium mevalonate. On day 3, 16 h after incubation, total RNAs from cells were prepared and subjected to reverse transcription and qRT-PCR as described in Materials and Methods. Each value represents the amount of mRNA relative to that in the cells transfected with miR-NC, which was arbitrarily defined as 1. miR-NC or NC denotes the negative control miRNA mimic. The values represents the mean ± SD (*P<0.05). (C) After cells were transfected and incubated as described Fig.2A, on day 2, cells were switched with 10% DLPS or 0.1 mM rosuvastatin and 0.05 mM sodium mevalonate, or 1 μg/ml 25-hydroxycholesterol and 10 μg/ml

cholesterol as indicated in the figure. After 16 h of incubation, on day 3, cells were harvested and immunoblot analysis was carried out as described for Fig. 1. (D, E) On day 0, HepG2 cells were transfected with the indicated concentration of miR-224, or miR-1912 on 12-well plates as described above. The total amounts of transfected miRNA mimics were adjusted to 80 nM per well with a miR-NC. Cells were incubated and harvested, and immunoblot analysis was carried out as described for Fig. 1. (F) On day 0, HepG2 cells were transfected with the indicated miRNA mimics at final concentration of 40 nM and the indicated antisense oligonucleotides against miRNAs (anti-miR) at final concentration of 100 nM as described above. Cells were incubated and harvested, and immunoblot analysis was carried out as described for Fig. 1.

4. miRNAs targets the 3′UTR of *PCSK9* mRNA

In order to verify whether miRNAs affect PCSK9 expression via the 3′UTR of *PCSK9* mRNA, the sequence used for predicting the miRNAs by TargetScan and miRanda analysis, luciferase reporter assay was carried out using pLightSwitch_PCSK9_3UTR which contains the entire 3′UTR of *PCSK9* mRNA flanked to the downstream of renilla luciferase reporter gene (Fig. 3A). Sequences of putative binding sites for miR-224 and for miR-1912 and for unrelated sites are depicted in Fig. 3B and Fig. 3D, respectively, and locations of introduced mutations are indicated. Co-transfection into HEK293 cells with the wild-type construct with miR-224 or miR-1912 resulted in decreased luciferase activity as compared to luciferase activity in cells co-transfected with the control miRNA mimic (WT in Fig. 3C, E). When mutations are introduced to putative binding regions for miRNAs in the 3′UTR by site-directed mutagenesis (Mut-1, -2, and -3 in Fig. 3C and 3E), the luciferase activity remained unchanged by miRNAs. However, introduction of the mutation at the region unrelated to the predicted binding region in the 3′UTR showed no effect on reduction of the wild-type reporter activity by miRNAs (Mut-4 in Fig. 3C and 3E). These results suggested that miR-224 and miR-1912 reduce the expression of PCSK9 by targeting the corresponding regions in *PCSK9* mRNA specifically.

B

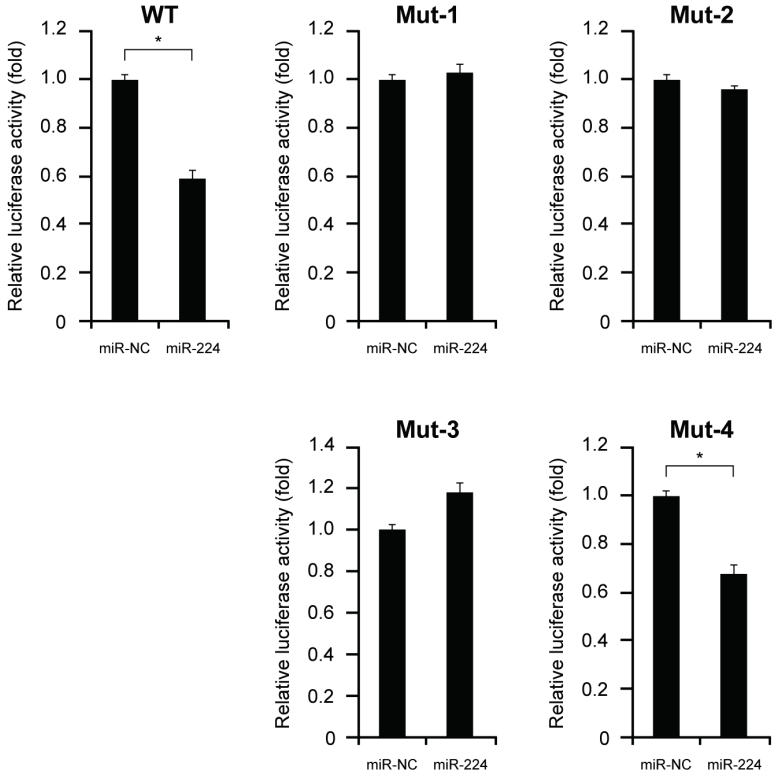
hsa-miR-224

3′-UUGCCUUGGUGAU**CACUGAA**C-5′

PCSK9 3'UTR reporter

WT	5′-ATTCTGGGTTTTGTAGCATTTTTATTAATATG**GTGACTT**TT-3′
Mut-1	5′-ATTCTGGGTTTTGTAGCATTTTTATTAATATG**GTGACT**GTT-3′
Mut-2	5′-ATTCTGGGTTTTGTAGCATTTTTATTAATATGT**TGAC**G**T**TT-3′
Mut-3	5′-ATTCTGGGTTTTGTAGCATTTTTATTAATATG**GTG**C**CTT**TT-3′
Mut-4	5′-ATTCT**T**G**T**TTTTGTAGCATTTTTATTAATATG**GTGACTT**TT-3′

C



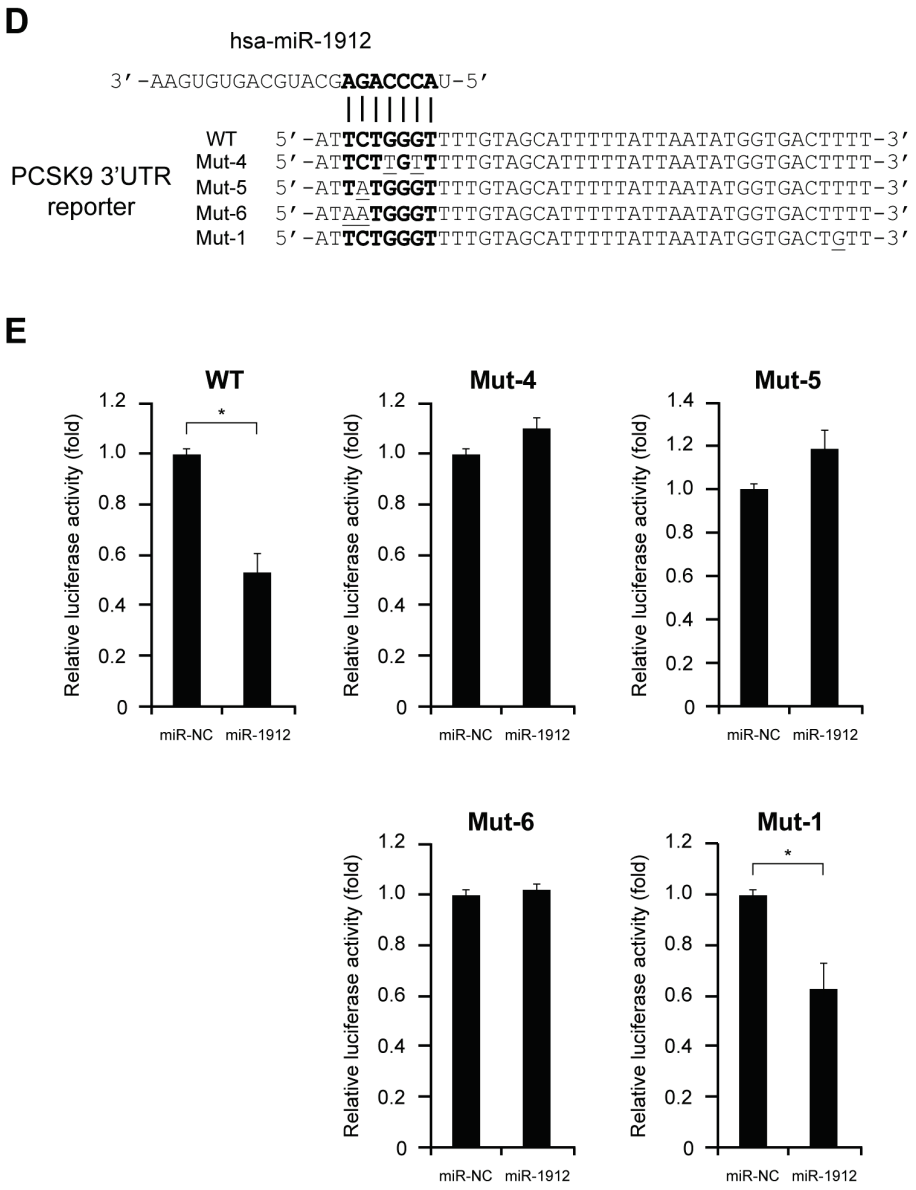


Figure 3. Specific interaction between selected miRNAs and the 3′UTR of *PCSK9* mRNA

(A) Schematic representation of the luciferase reporter vector harboring the *PCSK9* 3′UTR fragment (gray box). (B) Interaction between miR-224 and *PCSK9* 3′UTR predicted by TargetScan and miRanda. The interaction is highlighted in bold font. Point mutations were generated within the binding region by site-directed mutagenesis as described in Material and Methods, and are underlined. The name of each construct is shown at left. (C) Relative luciferase activities after co-transfection of *PCSK9* 3′UTR vectors with miR-224. HEK293 cells were co-transfected with pGL3 control, indicated *PCSK9* 3′UTR reporter, and indicated miRNA mimics as described in Material and Methods. After 48 h of incubation, cells were harvested and the luciferase activities were measured. The luciferase activities were normalized with firefly luciferase activities and the amounts of proteins used for assays. Each value represents the luciferase activities of indicated 3′UTR reporter constructs in the cells co-transfected with miR-224 relative to that in the cells co-transfected with miR-NC, which was arbitrarily defined as 1. Each value represents the mean ± SD (*P<0.05). (D) Interaction between miR-1912 and *PCSK9* 3′UTR predicted by TargetScan and miRanda. The interaction was highlighted in bold font and mutant vectors which have point mutations inside or outside the binding region are shown. (E) Relative luciferase activities after co-transfection of *PCSK9* 3′UTR vectors with miR-1912. The luciferase

activities of indicated 3′UTR reporter constructs in the cells co-transfected with miR-1912 were normalized to that in the cells co-transfected with miR-NC. Each value represents the mean ± SD (*P<0.05).

5. miRNAs induce the uptake of LDL-cholesterol

To investigate whether the change of PCSK9 expression by miRNAs affects cholesterol metabolism via LDLR, the effect of miRNAs on LDL-cholesterol uptake was evaluated. After miRNAs were transfected, cells were incubated in a media containing fluorescent-tagged LDL (Dil-LDL). Then, fluorescence microscopy images were obtained and the uptake of Dil-LDL was measured through FACS analyses. Compared to the miR-NC transfected cells, the miR-224 transfected cells contained more red Dil-LDL particles (Fig. 4A). When a histogram of fluorescence intensity was composed using FACS, the histogram of selected miRNAs transfected cells resulted in a shift-to-left, indicating a relative increase cellular LDL-cholesterol uptake (Fig. 4B). MiR-224 increased fluorescence intensity by 20% and miR-1912 demonstrated a relative increase in Dil-LDL uptake by 70% or greater (Fig. 4C).

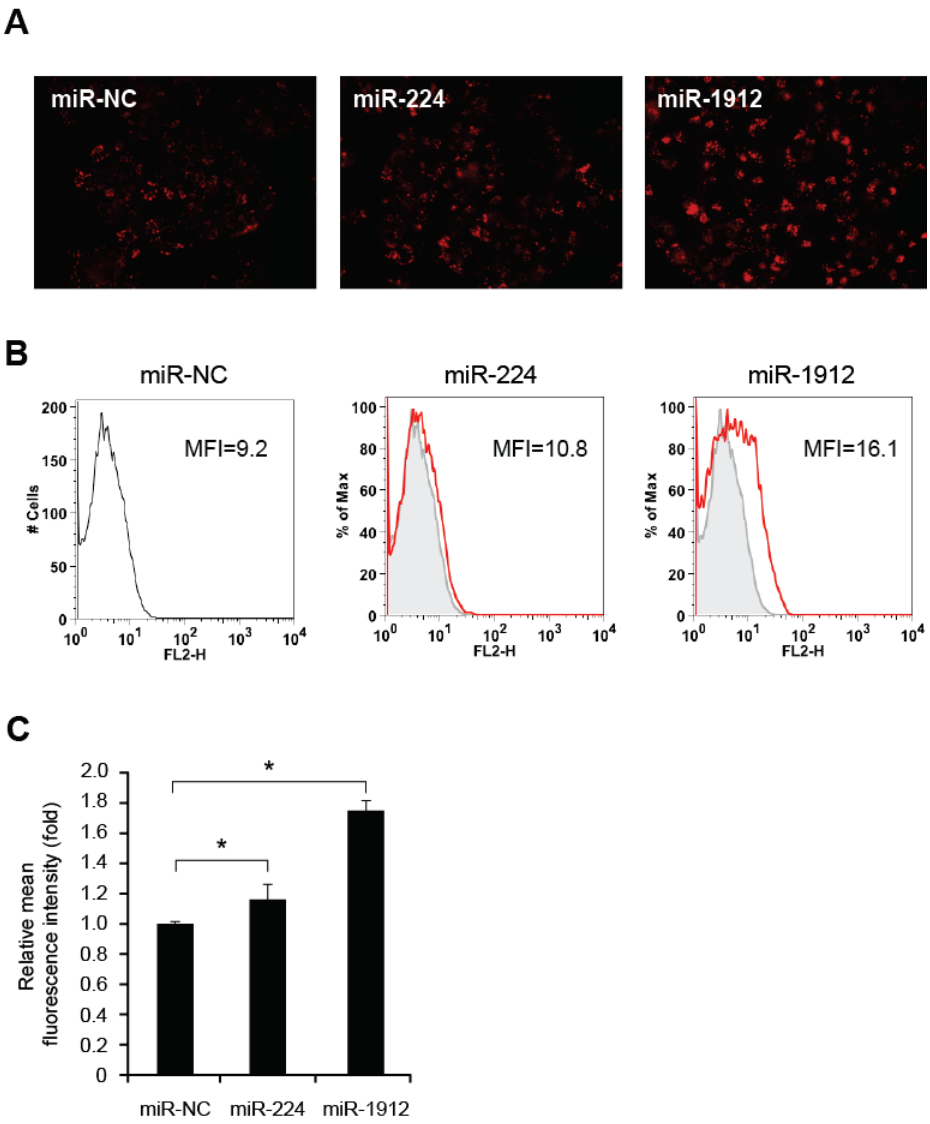


Figure 4. Enhanced cellular uptake of LDL-cholesterol by selected miRNAs

(A) Fluorescence microscopic images of Dil-LDL uptake in HepG2 cells

(x400). On day 0, HepG2 cells were transfected with miR-NC, miR-224 mimic, and miR-1912 mimic at final concentration of 40 nM as described in Material and Methods. On day 1, cells were switched to fresh medium A supplemented with 10% DLPS. On day 2, cells were switched to fresh medium A supplemented with 10% DLPS again. After 16 h of incubation, on day 3, cells were switched to fresh medium A added with 2 μg/ml of Dil-LDL. After 3 h of incubation, cells were washed twice with PBS and fluorescence microscopic images were taken. (B, C) After microscopic image acquisition, cells were trypsinized, and suspended in PBS, and analyzed with the flow cytometer. Mean fluorescence intensities (MFI) of 10^4 cells were analyzed by histogram and quantified. Histograms represent distribution of fluorescence intensities of Dil-LDL in cells transfected with miRNA mimics (red line). Diagrams show MFI of Dil-LDL in cells transfected with miRNA mimics relative to that in cells transfected with miR-NC. Each value represents the mean ± SD (*P<0.05).

6. Evaluation of physiologic regulation of selected miRNAs by cholesterol status in HepG2 cells

The expression of PCSK9 and LDLR is regulated by localization of SREBP-2 according to cellular cholesterol level. In order to evaluate whether these miRNAs have physiologic role to regulate cholesterol level, it was essential to validate the change of the endogenous expression of miRNAs targeting PCSK9 by cholesterol status. miR-224 resides in the intron 6 of gamma-aminobutyric acid (GABA) A receptor family epsilon subunit (GABRE) on the X chromosome (Fig. 5A). The putative miR-224 binding sites of PCSK9 3′UTR are highly conserved across species (Fig. 5B). In addition, the seed region of miR-224 are perfectly conserved (Fig. 5C).

After treatment of rosuvastatin or cholesterol to HepG2 cell to change cellular cholesterol content, qRT-PCR was employed to examine the expression of miR-224 and its host gene, *GABRE* (Fig. 6A, B). The amount of miR-224 was increased by cholesterol depletion. However, extracellular cholesterol induced the expression of miR-224 as well. Furthermore, the expression of miR-224 was increased more in response to simultaneous treatment of rosuvastatin and cholesterol. The amount of *GABRE* mRNA was also increased by treatment of rosuvastatin or cholesterol, but its change pattern was not similar to that of miR-224 according to cellular cholesterol

level.

Genomic locus of miR-1912 is in the intron 2 of the serotonin receptor 2C (5-hydroxytryptamine receptor 2C; HTR2C) on the X chromosome (Fig. 7A). The miR-1912 binding sites of PCSK9 3′UTR are conserved across only primates (Fig. 7B). Those of rodents are different in 2 nucleotides. The sequences, which are corresponsive to the seed region of human mature miR-1912, are conserved across primate. However, mature forms of miR-1912 are different in length among species (Fig. 7C).

The expression of miR-1912 and its host gene, *HTR2C* were examined by Taqman miRNA assay and qRT-PCR. They showed that Ct values were more than 35, suggesting miR-1912 and *HTR2C* did not express in HepG2 cells.

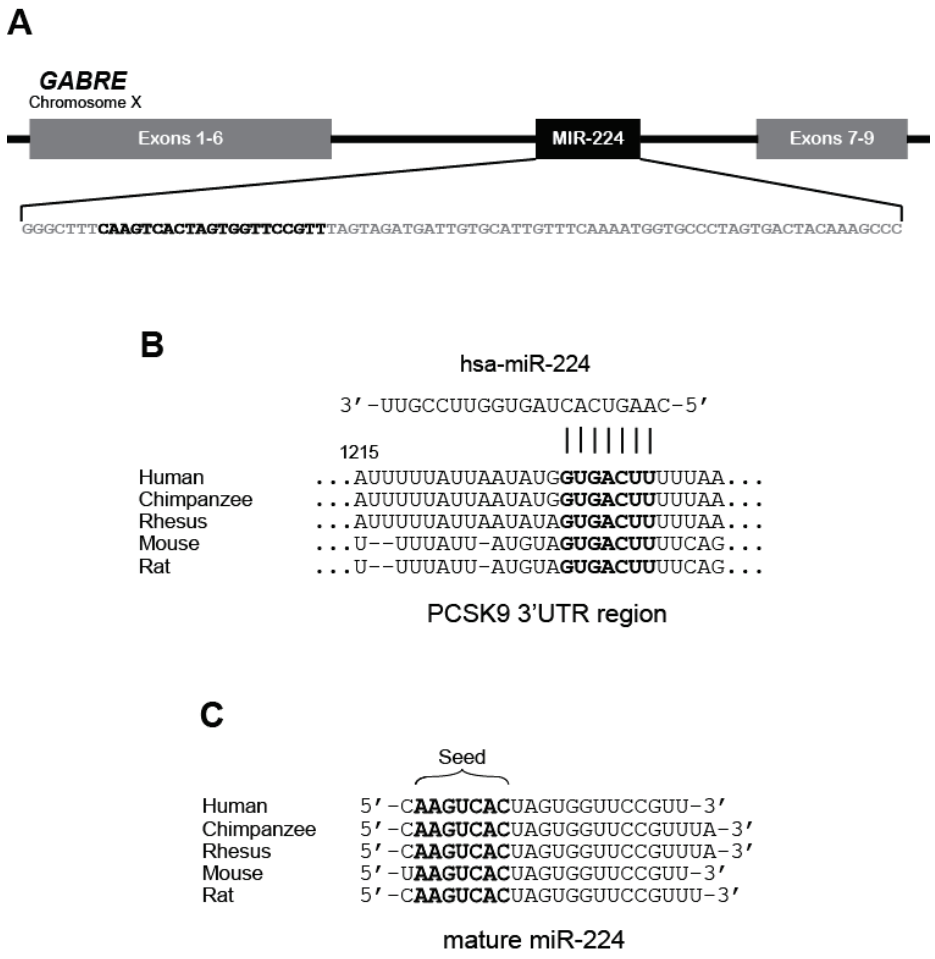


Figure 5. Genomic location of miR-224 and cross-species sequence conservation of miR-224 and its target site within the PCSK9 3′UTR

(A) Schematic representation of the miR-224 coding sequence within GABRE. The sequences encoding the pre-miRNA are shown, with the mature miR-224 sequences highlighted in bold. (B) 3′UTR sequences of *PCSK9*

flanking miR-224 target sites are aligned among human, chimpanzee, rhesus, mouse, and rat. Conserved sequences are shown in bold. (C) The sequences of mature miR-224 are aligned across species. Conserved sequences in the seed region are shown in bold. *GABRE,* gamma-aminobutyric acid A receptor family epsilon subunit.

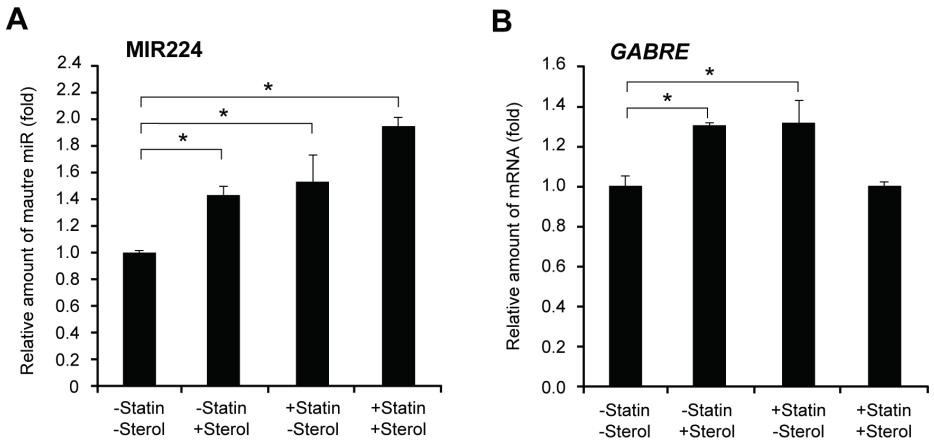


Figure 6. The expression of miR-224 and its host gene according to cellular cholesterol content

(A, B) Expression of miR-224 and its host gene, *GABRE* in HepG2 cells after depletion or supplementation of sterols. On day 0, HepG2 cells were set up with medium A supplemented with 10% FBS. On day 1, the cells were switched to fresh medium A supplemented with 10% DLPS for 24 h. On day 2, cells were switched with 10% DLPS or 0.1 mM rosuvastatin and 0.05 mM sodium mevalonate, or 1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol as indicated in the figure. After 16 h of incubation, on day 3, total RNAs from the cells were prepared and subjected to reverse transcription and qRT-PCR as described in Material and Methods. Each value represents the amount of miRNA or mRNA relative to that in the cells grown in the absence of rosuvastatin, mevalonate, and sterols. All the values are expressed as the

mean ± SEM (*P<0.05). *GABRE,* gamma-aminobutyric acid A receptor family epsilon subunit.

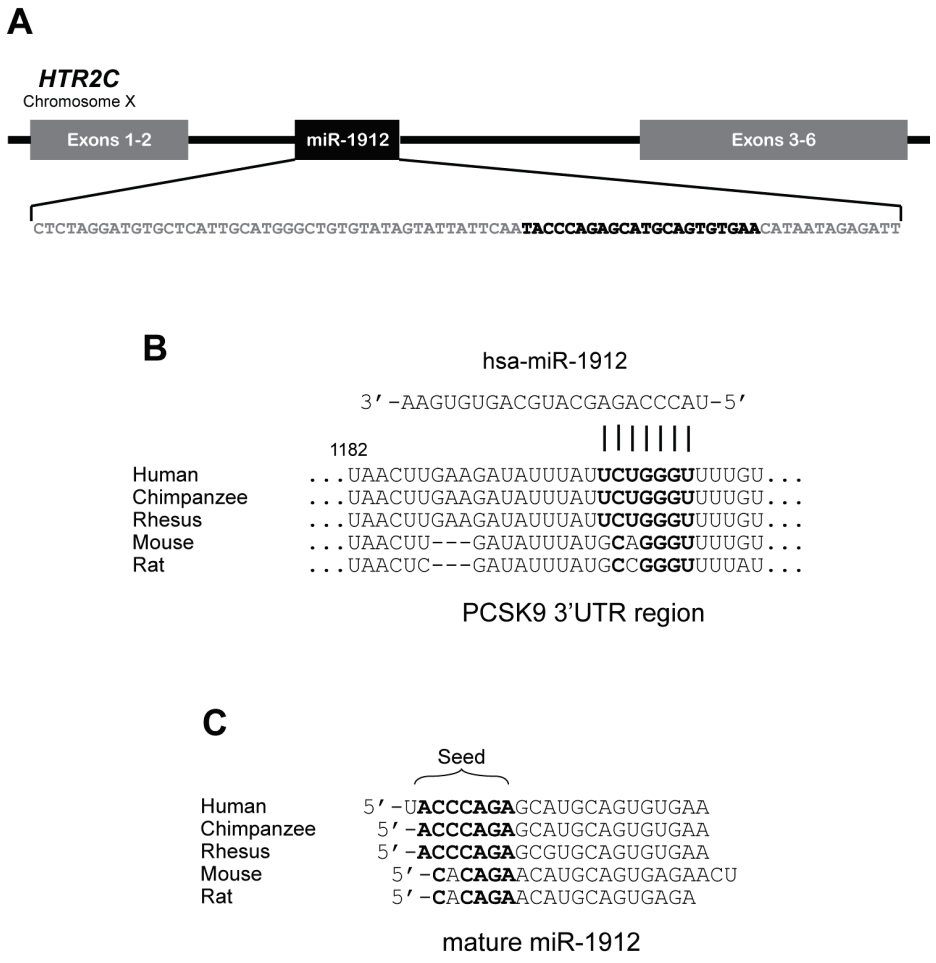


Figure 7. Genomic location of miR-1912 and cross-species sequence conservation of miR-1912 and its target site within the PCSK9 3′UTR

(A) Schematic representation of the miR-1912 coding sequence within HTR2C. The sequences encoding the pre-miRNA are shown, with the mature miR-1912 sequences highlighted in bold. (B) 3′UTR sequences of PCSK9

flanking miR-1912 target sites are aligned among human, chimpanzee, rhesus, mouse, and rat. Conserved sequences are shown in bold. (C) The sequences of mature miR-1912 are aligned across species. Conserved sequences in the seed region are shown in bold. *HTR2C*, 5-hydroxytryptamine receptor 2C.

7. Endogenous miR-224 affects the expression of PCSK9 and the uptake of cholesterol

Because miR-224 expressed in HepG2 cells, the effects of miR-224 knockdown on PCSK9 expression were examined. After anti-miR-224 transfection into HepG2 cells, the expression of mature miR-224 was examined by qRT-PCR. It showed that anti-miR-224 reduced endogenous miR-224 expression effectively (Fig. 8A) and increased *PCSK9* mRNA level significantly (Fig. 8B). In addition, immunoblot analysis revealed that anti-miR-224 increased the amount of PCSK9 and decreased the amount of LDLR (Fig. 8C).

To evaluate the relationship between increasing PCSK9 expression and endogenous miR-224 levels, 3′UTR luciferase assay was carried out after transfection of anti-miR in HepG2 cells. In the contrast with the results of miR-224 mimic on *PCSK9* 3′UTR reporter assay (Fig. 3C), anti-miR-224 increased the luciferase activity of *PCSK9* 3′UTR reporter in HepG2 cells. It suggests that the endogenous miR-224 regulates PCSK9 expression via specific miRNA-mRNA interaction.

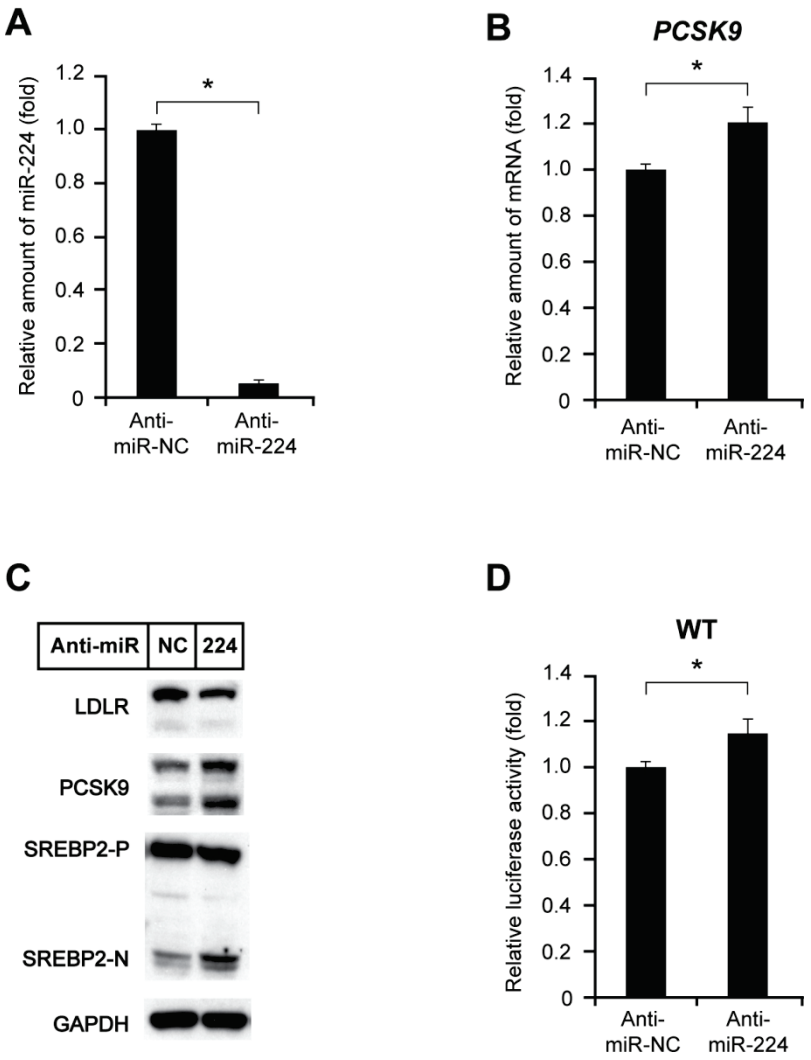


Figure 8. The effect of miR-224 inhibition on the expression of PCSK9 in HepG2 cells

(A, B) On day 0, HepG2 cells were transfected with anti-miR negative control (anti-miR-NC), and anti-miR-224 at final concentration of 80 nM as

described in Material and Methods. On day 1, cells were switched to fresh medium supplemented with 10% DLPS. On day 2, cells were switched to fresh medium A supplemented with 10% DLPS. After 16 h of incubation, on day 3, total RNAs from the cells were prepared and subjected to reverse transcription and qRT-PCR as described in Materials and Methods. Each value represents the amount of mRNA relative to that in the cells transfected with anti-miR-NC, which was arbitrarily defined as 1. The values represents the mean ± SD (*P<0.05). (C) On day 3, cells were harvested and immunoblot analysis was carried out as described for Fig. 1. (D) 3′UTR reporter assay was performed in HepG2 cells as described for Fig. 3.

8. miR-224 could regulates another LDL receptor degradation mechanism

TargetScan and miRanda also revealed that inducible degrader of the LDL receptor (IDOL), which induces the degradation of LDLR, could be another target of miR-224 (Fig. 9A). To evaluate the specific interaction between miR-224 and *IDOL* mRNA, which was predicted by bioinformatic algorithms, IDOL reporter assay was carried out. MiR-224 reduced the luciferase activity of WT reporter by 40%. However, the luciferase activities of reporters harboring mutated miR-224 binding site in *IDOL* 3′UTR were not changed by miR-224 (Fig. 9B). In order to evaluate endogenous miR-224 effects on *IDOL* 3′UTR, reporter vectors were transfected in cells without miR mimics. Interestingly, the luciferase activity of mutant vector harboring totally mutated miR-224 binding region was increased significantly compared to that of WT vector (Fig. 9C). It showed that endogenous miR-224 could regulate the IDOL expression.

For further evaluation of this possibility, IDOL protein expressions and miR-224 expressions were examined in human hepatocellular carcinoma sample as miR-224 is known to be increased in hepatocellular carcinoma.[20,21] In tumor tissues IDOL protein expressions were significantly decreased in immunoblotting analysis (Fig. 10A). Furthermore, qRT-PCR results showed

that miR-224 expression were up-regulated 17 folds on average in tumor tissues compared to those in paired normal tissues (Fig. 10B). IDOL protein levels were inversely correlated with miR-224 expression in human tissue samples ($r=-0.596$, $p=0.015$) (Fig. 10C).

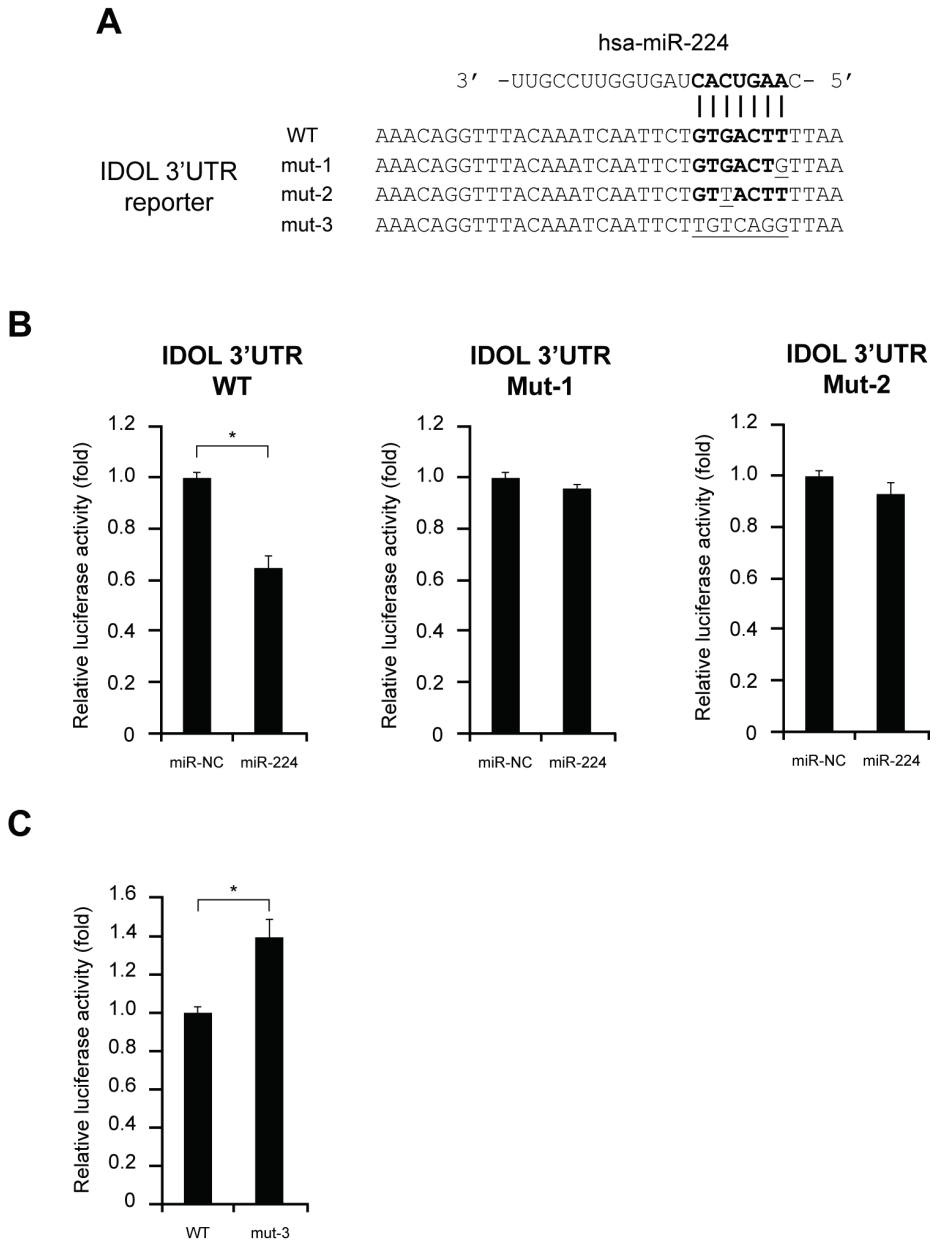
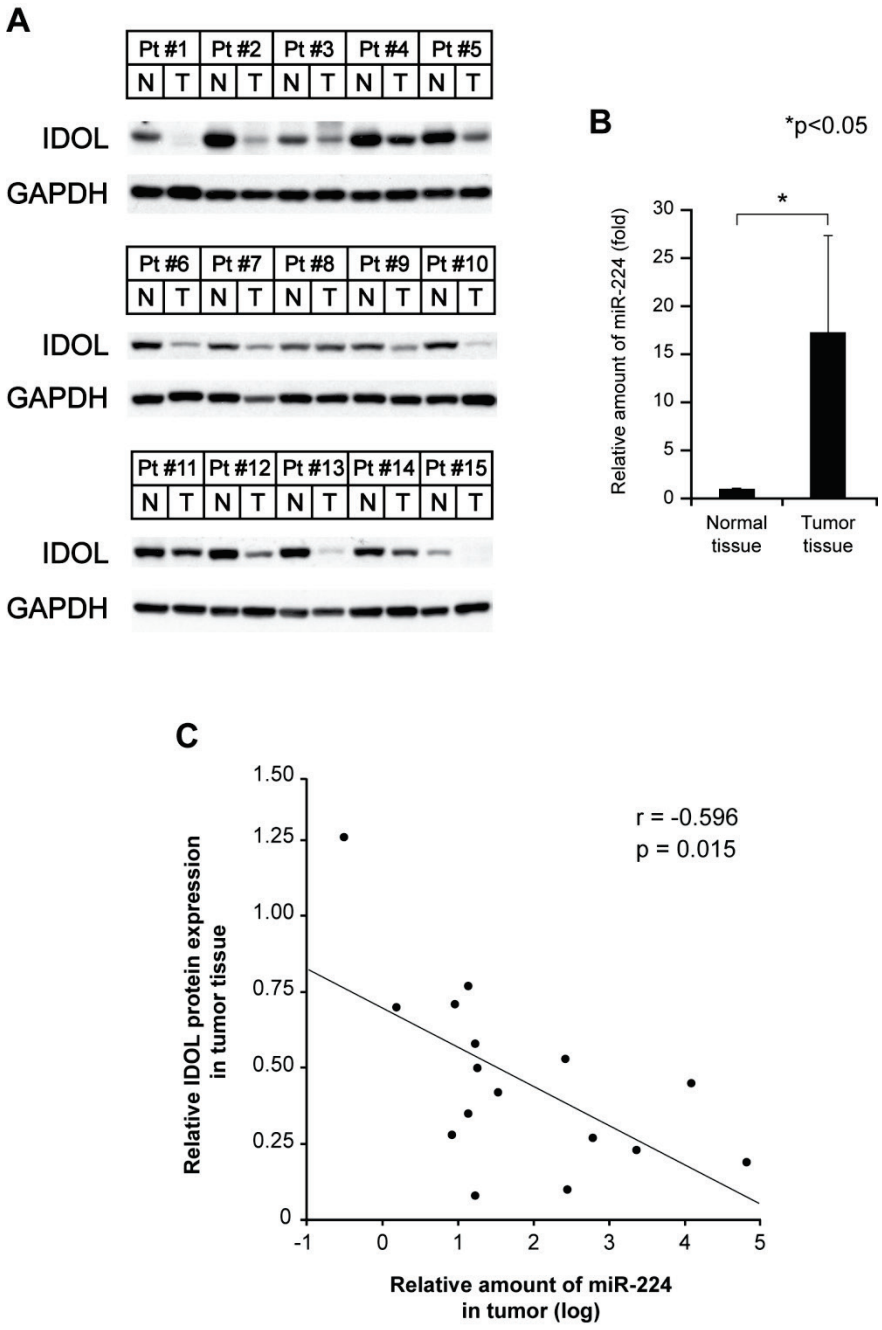


Figure 9. Specific interaction between miR-224 and 3′UTR of *IDOL*

(A) Interaction between miR-224 and *IDOL* 3′UTR predicted by TargetScan and miRanda. The interaction is highlighted in bold font. Point mutations within the binding region were generated by site-directed mutagenesis as described in Material and Methods, and are underlined. (B) 3′UTR reporter assays using WT or mutated vectors were performed as described for Fig. 3. (C) The luciferase activities of IDOL 3′UTR WT reporter vector and *IDOL* 3′UTR mut-3 reporter vector were compared in HepG2 cells. HEK293 cells were co-transfected with pGL3 control, and indicated *IDOL* 3′UTR reporter as described in Material and Methods. After 48 h of incubation, the luciferase activities were measured and are represented relative to that in the cells transfected with WT reporter. Each value represents the mean ± SD (*P<0.05).


A
Pt #1
Pt #2
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Pt #4
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N T N T N T N T N T
IDOL
GAPDH
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IDOL
GAPDH
Pt #11
Pt #12
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Pt #15
N T N T N T N T N T
IDOL
GAPDH
B
*p<0.05
Relative amount of miR-224 (fold)
30
25
20
15
10
5
0
*
Normal tissue
Tumor tissue
C
Relative IDOL protein expression in tumor tissue
1.50
1.25
1.00
0.75
0.50
0.25
0
r = -0.596
p = 0.015
-1
0
1
2
3
4
5
Relative amount of miR-224 in tumor (log)

Figure 10. The expression levels of miR-224 and IDOL in clinical specimens

(A) Protein expressions were examined by immunoblotting 15 pairs (N, normal; T, tumor) of liver samples. (B) miR-224 expression levels were quantified by qRT-PCR and those of tumor tissues normalized to those of normal tissues on average. (C) Protein levels were quantified with imageJ software. IDOL protein levels in tumor tissues were normalized to those in paired normal tissues. Correlation analysis of miR-224 expression and IDOL protein levels in tumors was performed (r=-0.596, P=0.015).

IV. DISCUSSION

Cholesterol is essential for most vertebrates as an important component in cellular membrane and a precursor in metabolic pathways, but excess of cholesterol can cause atherosclerosis and cardiovascular disease.[22] Therefore cholesterol homeostasis has to be strictly regulated in the body. Classical concept of regulating cholesterol metabolism is a coordination of 2 transcription factor, liver receptor X (LXR) and SREBP-2.[8,23,24] When excess cholesterol is accumulated in cells, LXR activates the expression of genes involved cholesterol efflux. By contrast, SREBP-2 induces cholesterol synthesis and uptake related genes.

A class of non-coding RNAs termed miRNAs has emerged as important modulators of numerous cellular processes that affect organism's growth, developments, homeostasis and disease.[25] Recently the role of miRNA in metabolic homeostasis has been studied. Several studies revealed that miRNA is a potent posttranscriptional regulator of cholesterol metabolism in addition to the classic transcriptional regulation.[26]

The purpose of this study was to discover miRNAs regulating PCSK9 and to investigate whether these miRNAs affect cellular cholesterol homeostasis or not. The miRBase database provides all miRNA sequences and information about novel miRNAs using small RNA deep sequencing data

sets.[27] In addition, multiple bioinformatic algorithms are available on the internet for identification of miRNAs targeting specific genes.[28,29] They have been the most useful tools in predicting miRNA targets. However, each algorithm often shows different prediction results of miRNA candidates for one gene. It is reason that each algorithm may predict distinct miRNA binding sites and they analyze probabilities of miRNA-mRNA interactions differently. Therefore, it is recommended that at least two algorithms should be for more predictive accuracy.[30]

In this study, 2 algorithms, TargetScan and miRanda, were used for prediction of miRNAs targeting PCSK9. Expectedly, both algorithms showed hundreds of miRNAs and their lists were different from each other in some degree. According to the probability score of functional miRNA-mRNA interactions, top 10 ranked miRNAs of both algorithms were compared and overlapped miRNAs were selected as potential regulators of PCSK9 for additional validation experiments.

Specificity of miRNA target selection is affected by various unknown factors besides seed paring.[31] Therefore experimental validation should be needed to confirm whether the predicted miRNAs are functional on certain biological process. For example, miR-99b, a top-ranked miRNA targeting PCSK9 in TargetScan, did not change the expression of PCSK9 (Fig. 1).

Hence among selected miRNAs, miR-224 and miR-1912 were finally selected through immunoblot results.

miR-224 and its predicted binding sites on *PCSK9* 3′UTR are well conserved among vertebrae (Fig. 5). Well conserved miRNAs are thought to be vital and evolutionary ancient component of genetic regulation.[32,33] For example miR-33 and its putative binding site in *ABCA1* are well conserved across the species. MiR-33 locates within intron 16 of human *SREBP-2*.[34] It has reported that *SREBP2* and miR-33 are coexpressed and regulated by the change of cholesterol level.[17] When intracellular cholesterol is depleted, the expression of SREBP2 is induced and the genes for cholesterol uptake or synthesis are activated by SREBP2. Simultaneously, increased miR-33 represses ABCA1, which transports sterols from cells and mediates high-density lipoprotein assembly, and functions in concert with its host gene to regulate cholesterol homeostasis.[14,17,34,35]

Therefore miR-224 was supposed to play an evolutionary important role in cholesterol metabolism. However, it was disconcerting result that miR-224 expression changed regardless of variation in cellular cholesterol (Fig. 6A). In this regards, the expression change of *GABRE* by cholesterol levels was evaluated but it was irrelevant to cellular cholesterol as well (Fig. 6B). These results were insufficient to support the role of miR-224 in cholesterol

metabolism regulation. Though, perhaps it is because most experiments were performed in HepG2 cell which miR-224 is pathologically increased.[36] Therefore, the more physiological condition should be needed to prove the role of miRNA in metabolic regulation.

In comparison with conserved miRNAs, poorly conserved miRNAs are thought to be less important in regulation pathway. While, the different number of miRNAs and diversity of miRNAs among species seem to be a vital evolutionary mechanism of phenotypic diversification and speciation.[37] miR-1912 locates on intron 2 of *HTR2C* that expresses exclusively in neuronal cells.[38] It is a quite impressive result which miR-1912 had great effect on cholesterol metabolism with significant decreasing PCSK9 and increasing LDLR. However, the function of endogenous miR-1912 on cholesterol metabolism could not be examined in this study because miR-1912 does not express in HepG2 cells. To this day, the expression of miR-1912 has been confirmed in just brain cortex and primary lymphocytes.[39] At first PCSK9 was identified as a molecule involving neuronal differentiation.[40] Therefore it is conceivable that miR-1912 can be related with tissue specific or species specific regulation of cholesterol metabolism.

It is known that single miRNA can target many genes.[12,25] Besides PCSK9, other genes which involve the regulation of the LDL receptor had

been searched for their possibilities as target of miR-224. Bioinformatic algorithms predicted that miR-224 also targets IDOL which induces the degradation of LDLR as it name indicates. From *IDOL* 3′UTR reporter assay, the specific interaction between miR-224 and *IDOL* mRNA was proved. Furthermore, it was confirmed that the expression of miR-224 was inversely correlated with IDOL protein level in human hepatocellular carcinoma tissues (Fig. 10). The plasma cholesterol levels are lower in patients with hepatocellular carcinoma than in healthy subjects, because hepatic cellular damage impairs the process of cholesterol synthesis or absorption, leading to change in plasma lipid pattern.[41] Recently several studies depict increase of miR-224 in hepatocellular carcinoma. miR-224 was found to influence migration and invasion of cancer cells during carcinogenesis.[21,42,43] This study identified that miR-224 regulates cellular cholesterol uptake partially by controlling the IDOL expression which regulate the degradation of LDLR. This supports that the hypocholesterolemic trait is common in hepatocellular carcinoma patients, and provides some insights in the role of miRNA on cholesterol metabolism in cancer biology.

V. CONCLUSION

When the predicted miRNAs was overexpressed, PCSK9 protein levels declined so that cellular cholesterol uptake rose. LDLR is in charge of cholesterol uptake and its degradation is regulated by PCSK9 and IDOL.[44,45] Up to this day, it is known that PCSK9 expression is controlled by transcription factors such as SREBP-2 or HNF-1.[46] The present study showed that miRNAs directly controls PCSK9 protein levels with specific miRNA-mRNA interactions. Especially the change of endogenous miR-224 levels affects PCSK9 proteins levels, as a result the amount of LDLR protein alters significantly. It would expand our knowledge of how PCSK9 is regulated besides transcriptional regulation mechanisms. Also miR-1912 showed noteworthy suppression of PCSK9 so it would be helpful to research the new PCSK9 inhibitors even though off-target effects of miR-1912 should be investigated.

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Abstract (in Korean)

마이크로RNA에 의한
PCSK9 및 콜레스테롤 항상성 조절

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이 찬 주

세포 표면의 저밀도 지단백 수용체는 혈중 저밀도 지단백 콜레스테롤 세포 내로 흡수하는 역할을 하며 혈중 농도를 조절하는데 중요하다. Proprotein convertase subtilisin/kexin type 9 (PCSK9)은 저밀도 지단백 수용체와 결합을 해 세포 내부로 이동을 시켜 제거하며 이를 통해 콜레스테롤 항상성을 조절한다. PCSK9의 전사는 스테롤 반응

요소 결합 단백질 2에 의해서 조절이 된다고 알려져 있으나 아직까지 마이크로RNA에 의한 조절기전은 밝혀진 바가 없다. 이 연구의 목적은 PCSK9을 조절하고 콜레스테롤 항상성에 영향을 끼치는 마이크로RNA의 존재를 밝히는 것이다. 마이크로RNA에 의한 PCSK9의 전사 후 조절기전을 밝히기 위해서 바이오인포매틱 알고리즘을 이용해서 PCSK9을 조절할 것으로 예측되는 마이크로RNA들을 스크리닝하였다. 예측된 마이크로RNA 중에서 miR-224와 miR-1912가 웨스턴 블롯 분석에서 PCSK9을 줄이고 저밀도 지단백을 증가시키는 것을 확인하였다. 3′ untranslated region (3′UTR) luciferase assay는 이 마이크로RNA들이 *PCSK9* mRNA의 3′UTR 에 특이적으로 작용한다는 것을 보여주었다. 형광 표식 저밀도 지단백 흡수 분석을 통하여 miR-224나 miR-1912가 세포의 저밀도 지단백 흡수를 70%까지 증가시키는 것을 확인하였다. 그리고 miR-224는 저밀도 지단백 수용체의 유도를 일으키는 또 다른 물질인 inducible degrader of the LDL receptor (IDOL)을 조절하는 것을 3′UTR luciferase assay를 통해 확인하였다. 이런 결과들은 마이크로RNA가 PCSK9이나 IDOL의 전사 후 조절을 통해 콜레스테롤 항상성을 조절하는 또 다른 기전일 가능성을 의미하며, 이런 점은 고지혈증 치료에 한가지 방법으로 응용할 가능성이

있음을 시사한다.

핵심되는 말: PCSK9, 저밀도 지단백 수용체, 마이크로RNA, 고콜레스테롤