Identification of miR-185 as a regulator of de novo cholesterol biosynthesis and low-density lipoprotein uptake

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Running Title: regulation of lipoprotein uptake by miR-185

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Abbreviations: SREBP, sterol response element binding protein; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; INSIG, insulin signaling gene; HDL-C, high density lipoprotein-cholesterol; SRE, sterol response element; SR-BI, scavenger receptor BI; HMGCR, HMG-CoA reductase, FDFT1, squalene synthase; MCD, methyl-beta-cyclodextrin; TSS, transcriptional start site; ChIP, chromatin immunoprecipitation; S1P, site-1 protease; S2P, site-2 protease; AMPK, Adenosine monophosphate kinase; PUFA, polyunsaturated fatty acid; LXR, liver X receptor; 25-HC, 25 hydroxycholesterol; CE, cholesterol ester; RCT, reverse cholesterol
transport; HPCD, hydroxypropyl-beta-cyclodextrin; MRE, miR response elements; italicized $SREBP-2$ represents the gene; normal text SREBP-2 represents protein; italicized $SREBP-1c$ represents the gene; normal text SREBP-1c represents protein.
ABSTRACT

Dysregulation of cholesterol homeostasis is associated with various metabolic diseases, including atherosclerosis and type 2 diabetes. The sterol regulatory element-binding protein 2 transcription factor (SREBP-2) induces the expression of genes involved in de novo cholesterol biosynthesis and low-density lipoprotein (LDL) uptake, thus it plays a crucial role in maintaining cholesterol homeostasis. Here, we found that overexpressing miR-185 in HepG2 cells repressed SREBP-2 expression and protein level. miR-185-directed inhibition caused decreased SREBP-2-dependent gene expression, LDL uptake, and HMG-CoA reductase activity. In addition, we found that miR-185 expression was tightly regulated by SREBP-1c, through its binding to a single SRE element in the miR-185 promoter. Moreover, we found that miR-185 expression levels were elevated in mice fed a high-fat diet, and this increase correlated with an increase in total cholesterol level and a decrease in SREBP-2 expression and protein. Finally, we found that individuals with high cholesterol had a 5-fold increase in serum miR-185 expression compared to control individuals. Thus, miR-185 controls cholesterol homeostasis through regulating SREBP-2 expression and activity. In turn, SREBP-1c regulates miR-185 expression through a complex cholesterol-responsive feedback loop. Thus, a novel axis regulating cholesterol homeostasis exists that exploits miR-185-dependent regulation of SREBP-2 and requires SREBP-1c for function.

Supplementary Keywords: microRNA, metabolism, cholesterol, transcription, SREBP
INTRODUCTION

Cholesterol is a key lipid of eukaryotic cell membranes and is an essential precursor for steroid hormone and bile acid syntheses. The dysregulation of cholesterol homeostasis is tightly associated with various metabolic diseases, including atherosclerosis and type 2 diabetes (1, 2). Besides taking up cholesterol in the form of apolipoprotein B-containing lipoproteins, particularly LDL by LDLR, animal cells *de novo* synthesize cholesterol from acetyl-CoA through a series of enzymatic reactions (1-7). Genes coding for enzymes involved in the *de novo* pathway are subject to transcriptional regulation by the SREBP family of transcription factors (8). SREBP family members are basic-helix-loop-helix leucine zipper transcription factors that bind to sterol response elements (SREs) and promote gene expression (9). SREBP-1a, SREBP-1c and SREBP-2 comprise the family and are encoded by two distinct genes, *SREBF-1* and *SREBF-2*. SREBP-2 is the main regulator of *de novo* cholesterol biosynthesis, controlling the transcription of cholesterol biosynthetic genes, including *HMGCR* and *FDFT1*, as well as, *LDLR* and *PCSK9* (8). On the other hand SREBP-1c targets genes involved in fatty acid metabolism (10). Although SREBP-1a regulates fatty acid and cholesterol related gene expression, SREBP-1c expression level predominates in the liver (11).

Intracellular cholesterol concentration is tightly regulated by feedback mechanisms controlling the cleavage/activation and nuclear translocation of SREBPs. When the level of cholesterol is high, SREBP-2 is bound to a SCAP-INSIG complex in the ER (8), whereas when the level of cholesterol decreases, SCAP disassociates from INSIG and guides the translocation of SREBP-2 from the ER to the golgi (8, 12). There in the golgi, full-length SREBP-2 is cleaved
by S1P/S2P and converted to a soluble mature SREBP-2 transcription factor that translocates to
the nucleus, where it binds SREs in the promoters of sterol-responsive genes (12, 13).

Elucidating the mechanisms controlling the expression and activation of SREBP-2 is key
to our understanding of cholesterol metabolic regulation. The events regulating the
transcriptional expression of SREBP-2, and the post-translational modifications affecting
SREBP-2 activity are well documented (14-26). On the other hand, microRNA-dependent
SREBP-2 post-transcriptional regulation is only now being explored. MicroRNAs (MiRs) are
~22 nucleotide single-stranded, small, non-coding RNAs. MiRs can suppress gene expression
post-transcriptionally by imperfect pairing to MiR Response Elements (MREs) within 3’ UTRs
of target mRNAs. MiR regulation results in the inhibition of target gene expression through
mRNA degradation and/or translational inhibition (27, 28). MiRs are transcribed from locations
throughout the genome within the introns and exons of protein-coding genes, as well as, multiple
intragenic regions. MiRs can be transcriptionally regulated by changes in promoter activity (29-
34). The aberrant expressions of certain miRs are associated with cardiometabolic diseases (27,
35, 36).

miR-185 and its role in cell biology first came to light when a connection was discovered
between miR-185 expression and cancer progression. For instance, miR-185 overexpression
suppressed the migration and invasiveness of LNCaP prostate cancer cells (37), while its
repression led to cisplatin resistance in SKOV3/DDP ovarian cell lines (38). Moreover, specific
miR-185 SNPs have an inverse relationship with breast cancer risk, suggesting this miR may
have biomarker attributes (39). miR-185 targets include RhoA, Cdc42, DNA methyltransferase
1, the androgen receptor, and the Six1 oncogene (37, 40-42). While evidence is accumulating
establishing the miR-185 cancer connection, its role in regulating lipid metabolism is obscure.
Very recently, miR-185 was shown to repress selective HDL-C uptake through the inhibition of SR-BI expression in human hepatic cells (43). This is the only report linking miR-185 to any type of lipid metabolic regulation. Interestingly, another miR, miR125a-5p, was also found to inhibit SR-BI expression in steroidogenic cells (44). We tested several miRs (miR-1260/532/324/185) potentially targeting the 3’UTR of SREBP-2 for the ability to modulate SREBP-2 expression. Of those tested, only miR-185 was found to significantly reduce SREBP-2 expression. Here, studies show that miR-185 regulates SREBP-2 expression, which in turn regulates LDLR expression and LDL uptake. Moreover, evidence is presented for the first time showing that SREBP-1c regulates miR-185 expression, thus there is a feedback loop that precisely regulates miR-185 activity in order to maintain cholesterol homeostasis.

MATERIALS AND METHODS

Cell Lines and Reagents.

THLE-2, HepG2, and 293T cells were obtained from ATCC. THLE-2 cells were cultured in BEGM medium with supplements (Lonza) following the manufacturer’s protocol. HepG2 cells were cultured in MEM medium (supplemented with 10% fetal bovine serum, 1% non-essential amino acid, 1% sodium pyruvate, 1% L-glutamine). 293T cells were cultured in DMEM medium (supplemented with 10% fetal bovine serum, 1% L-glutamine). Cells were incubated at 37 °C with 5% CO₂. For methyl-beta-cyclodextrin (MCD, Sigma) treatment, cells were incubated in serum free medium containing 50mM MCD at 37 °C for 10 minutes or 30 minutes (HMGCR
activity assay). 25-hydroxycholesterol (Sigma) was used at 2µg/ml for 6 hours. Insulin (Sigma) was used at 100nM.

**RNA Isolation and Quantitative Real-time PCR.**

Total RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA using RT Easy First Strand Kit (Qiagen). qRT-PCR was carried out using a Stratagene MX3005P (Stratagene). The relative mRNA levels were normalized to levels of GAPDH. In addition, small RNA was converted to complimentary DNA from 500ng of total RNA using the miScript II RT kit (Qiagen). miR level was determined by qRT-PCR using miR specific (miR-185) primers (Qiagen) and normalizing to RNU6-2 snRNA levels as a control. For miR-185 levels in mouse liver, miR-185 expression was normalized to SNORD66 level. Data is shown as the ratio of miR-185 expression as compared to either RNU6-2 or SNORD66.

**Transfection and Luciferase Reporter Assay.**

For human miR-185 over-expression, pre-miR-185 (Life Technology) was used. pLightswitch-SREBP-2 3’UTR luciferase reporter plasmid was obtained from Switchgeargenomics. 3’UTR mutant pLightswitch SREBP-2 reporter plasmid contained point mutations within miR-185 targeting sites (described in the results). The Quick Change Lightning Mutagenesis kit (Stratagene) was used to construct the SREBP-2 3’UTR mutant. Site insertion was confirmed by DNA sequencing. SREBP-1c siRNA and control siRNA were obtained from Thermo Scientific. Cells were incubated in serum free medium overnight prior to being transfected with pre-miR-
185, control pre-miR, Lightswitch-\textit{SREBP-2} 3’UTR reporter plasmid, Lightswitch-\textit{SREBP-2} 3’UTR mutant plasmid, \textit{SREBP-1c} siRNA, or control siRNA using Lipofectamine 2000 (Invitrogen), following the manufacturer’s protocol. Luciferase activity was detected using the Lightswitch Reporter Assay System (Switchgeargenomics) 24h after the transfection as described in the manufacturer’s protocol.

\textbf{Western Blotting.}

Western blotting was performed using anti-SREBP-2 rabbit polyclonal antibody (Abcam), anti-SREBP-1C rabbit polyclonal antibody (Santa Cruz) and anti-LDLR rabbit polyclonal antibody (Abcam). Anti-β-actin mouse monoclonal antibody (Abcam) was used as the loading control. Secondary antibodies used were sheep anti-mouse HRP whole IgG, and donkey anti-rabbit HRP whole IgG (GE Healthcare).

\textbf{Immunocytochemistry Staining.}

48 hours after HepG2 cells were transfected with pre-miR-185 and control pre-miR, cells were fixed and labeled with rabbit anti-LDLR antibody (Cayman Chemicals, Ann Arbor, MI), followed by incubation of Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen). After antibody incubation, cells were mounted with fluorescent mounting medium containing DAPI (Invitrogen) for counter staining. Fluorescent microscopy was performed using a 20X objective on a Leica DMI6000 confocal microscope, and images were processed using LAS AF software.
LDL Uptake Assay.

24 hr after HepG2 cells were transfected with pre-miR-185 and control miR, cells were washed with PBS and incubated over night in lipoprotein-deficient medium (LPDS) to induce the expression of LDLR. LDL uptake was initiated by incubating cells that were grown in serum-deficient medium with 5 µg/ml BODIPY-LDL (Invitrogen). Uptake of BODIPY-LDL was measured after a 30 min incubation, at 37° C. Cells were fixed and mounted with fluorescent mounting medium containing DAPI (Invitrogen) for counter staining. Intercellular BODIPY-LDL was visualized using fluorescence microscopy.

HMGCR Activity Assay.

HMGCR activity assay was performed as described previously (45, 46). Briefly, 24 hours after HepG2 cells were transfected with pre-miR-185 or control pre-miR, cells were incubated with 5% LPDS over night to stimulate HMGCR activity. After lysing the cells, 100µg of total protein was incubated at 37° C for 30min in reaction buffer (20mM glucose-6-phosphate, 0.7 unit glucose-6-phosphate dehydrogenase, 3mM NADPH and 5mM DTT). The reaction was started by addition 30µM 14C-HMG-CoA (American Radiolabeled Chemicals). After a 2hr incubation at 37° C, the reaction was stopped by addition of 5N HCl (EMD) and 3µM 3H-mevalonolactone (American Radiolabeled Chemicals). 14C-mevalonolactone was separated from unreacted 14C - HMG-CoA by column chromatography using AG1-X8 resin (200-400 mesh, Millipore). After the samples were added to the resin bed, seven 1ml aliquots of water were used to elute 14C-mevalonolactone. The first 2ml of aliquots were discarded, and the next 5ml of elute were
quantified using Liquid Scintillation Counter (Beckman Coulter). HMG-CoA reductase activity was determined by normalizing isolated $^{14}$C-mevalonolactone from the internal control, $^3$H-mevalonolactone.

**Chromatin Immunoprecipitation.**

ChIP was performed using the Imprint Chromatin Immunoprecipitation Kit (Sigma) following the manufacturer’s instructions. Immunoprecipitation was performed using anti-polyclonal rabbit SREBP-1c (Santa Cruz) antibody and rabbit IgG (Santa Cruz) as a control. PCR was used to amplify the promoter region of miR-185 using primers detecting the 50bp-500bp upstream from TSS (Forward: ATCCAGAGTAAAGGCAGATAAGG and Reverse: GCGGAGACATGTCATCTCC).

**Non-radiolabeled Electrophoretic Mobility Shift Assay (EMSA) and Gel Super-shift.**

Nuclear extracts were prepared from HepG2 cells using the Nuclear Extract Kit (Active Motif, Carlsbad, Ca) as described by the manufacturer. Wild-type (WT) and mutant probes were synthesized as single stranded oligonucleotides with Biotin 3’-end labeling (Integrated DNA technology) from the -100 to -139 and -225 to -261 region of the miR-185 putative promoter. The sequences of the probes used were: Site 1 (putative SRE): WT Forward 5’- CAG CAG CCT GGG TAC TCA CCT GAG GTT ATT AGA CAG C -3’ and Reverse 5’- GCT GCT GTC TAA TAA CCT CAG GTG AGT ACC CAG GCT GCT G-3’ ; Mutant Forward 5’- CAG CAG CCT GGG TAC CGG CAG C -3’ and Reserve 5’- GCT GCT
GTC TAA TAA CCT **CCT GCC GGT ACC CAG GCT GCT G-3’**; Site 2 (putative SRE):
Forward 5’- GAG GCT GGA GCT CTC AGG CCA CCT GCC CAG GGC GAC TCC C -3‘ and
Reverse 5’- GGG AGT CGC CCT GGG CAG GTG GCC TGA GAG CTC CAG CCT C -3’;
Mutant Forward 5’- GAG GCT GGA GCT C**CTC** GGC CTC CAG GGC GAC TCC C -3’ and Reverse 5’- GGG AGT CGC CCT GGG CAG GGC GAC TCC C -3’ (potential binding sites shown in underline, mutated bases shown in bold text).

EMSA binding reactions were performed at room temperature for 20 min and consisted of nuclear extract in 1X binding buffer (50% glycerol, 100 mM MgCl2, 1µg/µl Poly (dI–dC), 1% NP-40, 1 M KCl, 200 mM EDTA and 5 µM DNA probe). The mixture was run on 6% non-denaturing polyacrylamide gels in 0.5X Tris Borate-EDTA buffer. Protein–DNA complexes were then transferred to Hybond-N+ nylon membrane using the Trans-Blot semi-dry method (Bio-Rad, CA), and cross-linked using the Spectrolinker XL-1000 UV crosslinker (Spectronics Corporation, NY). Detection of biotin-labeled DNA was performed using the LightShift chemiluminescent EMSA kit (Thermo Scientific, 20148) and visualized by exposure to a charge couple device camera (GE ImageQuant LAS 4000).

For EMSA competition studies, 20-fold molar excess of WT non-biotin labeled site 1 forward and reverse oligonucleotides were added to the EMSA reaction mix. For the gel-Supershift assay, following the incubation of the nuclear extracts with site 1 WT miR-185 promoter probes, 4µg of SREBP-1c mouse antibody (Santa Cruz) or 4µg of GAPDH mouse control antibody (GeneTex Inc., Irvine, Ca) were added to the reaction mixture, and incubated at room temperature for 30 min. The mixture was fractionated on a 5% non-denaturing polyacrylamide gel. Transfer and detection was performed as described above.
LXR activation assays.

HepG2 cells were treated with 1% HPCD (hydroxypropyl-beta-cyclodextrin) in serum free medium for 1hr at 37°C to remove intracellular cholesterol (47). To activate LXR signaling, cells were incubated with 2µg/ml 25-HC in serum free medium for 6hr at 37°C. qRT-PCR was then used to compare *SREBP-1c*, *ABCA1*, *ABCG1*, *SREBP-2* and miR-185 expression levels. GAPDH and RNU6-2 were used as internal controls for mRNA and miR, respectively. 25-HC was dissolved in ethanol, and control cells were added with the same volume of ethanol as control.

Insulin treatment.

HepG2 cells were grown in serum free medium with or without 100nM insulin overnight at 37°C. *SREBP-1c*, *SREBP-2* and miR-185 expression levels were determined by qRT-PCR. GAPDH and RNU6-2 were used as internal controls for mRNA expression and miR-185 expression, respectively. SREBP-1c protein level was examined by western blotting. GAPDH was used as internal control. Densitometry was used to normalize both full length and mature forms of SREBP-1c protein normalized to GAPDH protein level. The values are the average of three independent experiments.
Mouse feeding and lentiviral injection studies.

Wild type male C57BL/6J (B6) mice were purchased from Jackson Laboratories and housed at Temple University, Philadelphia, PA. Temple University Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures. 6-8 weeks old male B6 mice were fed either a normal diet (7% fat; BioServ, NJ) or a high fat diet (21% fat; BioServ, NJ) for 16 weeks. Fasted blood samples were taken every four weeks. Blood serum was obtained and used to measure total cholesterol using the Total Cholesterol Kit (Stanbio, Borerne, TX) following manufacturer’s protocol. Mice from each group were also sacrificed at weeks 4, 8, 12 and 16, and liver tissue was collected for protein and miR analysis.

6-8 weeks old WT male B6 mice were injected with $5 \times 10^9$ pfu lentivirus with or without primary miR-185 sequences, via retro-orbital injection. Two weeks post injection, mice were sacrificed and liver tissue was collected for miR analysis. Primary miR-185 sequence was cloned into pCDH-CMV-MCS-EF1 lentivector (SBI, Mountain view, CA). Lentivirus was generated by transfection of packaging plasmids (SBI, Mountain View, CA) and expressing the vector into 293T cells. The virus was collected 48hr post transfection.

Human Serum Samples.

Human normal serum samples (n=46) and hypercholesterolemia serum samples (n=40) were collected from Bioreclamation. All serum samples were from patients >50 years old. Mixed sex and race samples were used. Total RNA was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized from total RNA using RT Easy First Strand Kit (Qiagen In addition, small RNA
was converted to complimentary DNA from 100ng (human serum) of total RNA using the miScript II RT kit (Qiagen). miR-185 level was determined by qRT-PCR using miR specific (miR-185) primers (Qiagen) and normalizing to RNU6-2 snRNA level as a control. All patients were being treated with statins. Patients diagnosed with hypercholesterolemia had total cholesterol values > 240mg/dl and LDL levels >160mg/dl.

**Statistical Analyses.** The data shown are the average of at least three independent experiments. The data are the mean ± s.e.m. Statistical analysis was performed using *student’s t-test.*

**RESULTS**

**miR-185 expression is regulated by changes in cholesterol level.**

To begin to elucidate the role of miR-185 in cholesterol homeostasis, it was first addressed whether miR-185 expression was regulated in response to cholesterol level. HepG2 cells were treated with and without the cholesterol-depleting agent, MCD, and SREBP-2 and miR-185 expressions were determined. In the presence of MCD, SREBP-2 expression increased ~2.5-fold when compared to control cells (Fig. 1A, *Con vs. MCD (SREBP-2)*). This induction coincided with the cleavage of full-length SREBP-2 to the mature SREBP-2 fragment (Fig. 1B). Several groups have observed the cleavage and activation of SREBP-2 within this same timeframe (48, 49). miR-185 expression decreased by ~80% under the same conditions (Fig. 1A, *Con vs. MCD (miR-185)*).
miR-185 represses SREBP-2 expression through targeting SREBP-2 mRNA 3’UTR.

There are four predicted MREs within the 3’UTR region of human SREBP-2 mRNA, based on bio-informatics searches using TargetScan6.1 and miRBase (Fig 2A). To examine whether miR-185 targets to these predicted MREs, a luciferase reporter assay was used. HEK293 cells were transfected with a pSwitchLight WT-SREBP-2-3’UTR luciferase plasmid or a mutant SREBP-2-3’UTR luciferase reporter that has C to G point mutations in the four predicted miR-185 seeding sites: 1) CTCT(C→G) TCT(C→G) CCC; 2) TCT(C→G)T(C→G)T(C→G)CTG; 3) ACT(C→G)T(C→G)T(C→G)CTT; 4) ATT(C→G)T(C→G)T(C→G)CCT (Fig. 2B). WT-SREBP-2-3’UTR and Mut-SREBP-2-3’UTR luciferase activities were assayed in the presence of miR-185 or a control miR. The level of miR-185 increased ~10-13 fold.

Transfection of pre-miR-185 significantly decreased the cellular luciferase activity of cells carrying a wild type SREBP-2 3’UTR reporter plasmid (70%), as compared to cells transfected with only a control miR (Fig. 3A, D vs. E). The addition of point mutations within the four MREs significantly interfered with miR-185 targeting and silencing of SREBP-2 3’UTR luciferase activity (Fig. 3A, E vs. G). These results suggested that miR-185 interacted with the MREs found within the SREBP-2 mRNA 3’UTR.

Previous studies have shown that by targeting mRNA 3’UTRs, miRs silence target genes through initiating mRNA degradation or inhibition of translation (27, 28). To determine if miR-185 targets the 3’ UTR of endogenous SREBP-2 mRNA, miR-185 was tested for its ability to attenuate endogenous SREBP-2 mRNA and protein expression. HepG2 and THLE-2 cells were
transfected with pre-miR-185 or a control miR, and SREBP-2 mRNA expression was determined by qRT-PCR. Overexpression of miR-185 significantly decreased SREBP-2 mRNA level by ~60% in HepG2 and THLE-2 cells, as compared to miR-control cells (Fig. 3B, C). Addition of a miR-185 antagomiR abolished miR-185 targeting of the SREBP-2 3’UTR (Fig. 3B, C). The interaction of miR-185 to the endogenous SREBP-2 3’ UTR also reduced the level of SREBP-2 full-length and mature proteins (Fig. 3D). We do note that in both cell lines the addition of antagomiR plus miR-185 did not cause a further increase in SREBP-2 expression compared to control cells. It does suggest that endogenous miR-185 level is low in our cell lines. Overall, miR-185 negatively regulates SREBP-2 expression as a result of interaction with the SREBP-2 mRNA 3’UTR region, which leads to reduced SREBP-2 mRNA and protein level.

miR-185 affects SREBP-2-dependent gene expression.

SREBP-2 plays a critical role in regulating cholesterol metabolism by controlling the expression of several important genes involved in de novo cholesterol biosynthesis and LDL uptake. In order to examine whether miR-185 affects SREBP-2-dependent gene expression, HepG2 cells were transfected with pre-miR-185 or a control pre-miR and the mRNA expressions of HMGCR, FDFT1, and LDLR were determined by qRT-PCR. HepG2 cells expressing pre-miR-185 were treated with MCD. HMGCR, FDFT1, and LDLR expressions were determined in the absence or presence of MCD.

The levels of HMGCR and FDFT1 expressions did not significantly decrease in HepG2 cells over-expressing miR-185 (Fig. 4A & C vs. con miR vs. miR-185), while LDLR expression decreased by 60% (Fig. 4B, con miR vs. miR-185). The addition of MCD resulted in HMGCR up
regulation only in control cells (Fig. 4A, *con miR* vs. *Con miR + MCD*, >2.5-fold), while miR-185 over expression abolished MCD-stimulated *HMGCR* up regulation (Fig. 4A; *Con miR + MCD* vs. *miR-185 + MCD*). In the presence of a control pre-miR, *LDLR* and *FDFT1* gene expressions increased in the presence of MCD by >13-fold and >3-fold, respectively (Fig. 3B, C, *Con miR* vs. *Con miR + MCD*). The expression of miR-185 abolished MCD-stimulated *LDLR* and *FDFT1* expressions (Fig. 4B, C; *Con miR + MCD* vs. *miR185 + MCD*). These results indicate that miR-185 has a critical role in regulating cholesterol metabolism-related gene expression, via SREBP-2 post-transcriptional repression.

**miR-185 reduces LDLR protein, decreases LDL uptake, and attenuates HMGCR activity.**

The LDLR plays a critical role in LDL uptake by internalizing LDL-cholesterol via endocytosis (3,4). In order to explore the physiological significance of miR-185-dependent regulation of *SREBP-2* expression, the endogenous level of LDLR protein was examined by western analysis and indirect immunofluorescence, and LDL uptake was determined using the fluorescently-labeled LDL, LDL-BODIPY.

LDLR protein level was decreased in HepG2 cells over expressing pre-miR-185 as compared to pre-miR control cells (Fig. 5A). This result correlates with the expression data obtained (Fig. 3B). Moreover, the endogenous level of LDLR was drastically reduced in these cells (Fig. 5B; *Con miR/LDLR* vs. *miR-185/LDLR*). Finally, the level of internalized LDL-BODIPY was severely reduced in miR-185 expressing cells (Fig. 5C; *Con miR/LDL-BODIPY* vs. *miR-185/LDL-BODIPY*). Thus, miR-185 reduces cellular cholesterol level by reducing the uptake of LDL via decreased LDLR expression.
HMGCR is the rate-limiting enzyme of de novo cholesterol biosynthesis and converts HMG-CoA to mevalonate (50, 51). As demonstrated above, miR-185 inhibited MCD-induced HMGCR transcription through repressing SREBP-2 expression (Fig. 4A). To determine the physiological significance of this repression, HMGCR activity was determined in HepG2 cells overexpressing miR-185. In the presence of MCD, there was a 1.75-fold increase in HMGCR activity over baseline (Fig. 6, con vs. con + MCD). This increase was abolished when miR-185 was overexpressed (Fig. 6, con + MCD vs. miR-185 + MCD).

Transcriptional regulation of miR-185 by SREBP-1c.

Transcriptional regulation of miRs is a mechanism by which endogenous miR expression and function are regulated. The TSS (transcriptional start site) of pre-miR-185 was determined using miRStart (mirstart.mbc.nctu.edu.tw) (52), and 500bp upstream of the TSS was chosen to study miR-185 promoter activity. Two putative SREBP-1c binding sites are located within this promoter region (Fig. 7A; SRE1 & SRE2); the consensus sequence is TCACNCCAC. Based on the presence of these binding sites, SREBP-1c was tested for its ability to mediate miR-185 promoter activity. Interestingly, siRNA knock down of SREBP-1c in HepG2 cells (Fig. 7B) increased SREBP-2 mRNA (Fig. 7C) and protein (Fig. 7D) level. On the other hand, mature miR-185 expression level decreased in SREBP-1c knockdown HepG2 (>2-fold Fig. 7E) and THLE-2 cells (>2 fold, Fig.7F), as compared to control cells (con). These findings strongly suggested that SREBP-1c negatively regulated SREBP-2 expression through up regulating miR-185, possibly via binding to the promoter of miR-185. To test this hypothesis, chromatin immunoprecipitation (ChIP) was used to examine the binding of SREBP-1c to the miR-185
promoter. SREBP-1c was found to bind to the 500bp fragment upstream of the miR-185 TSS (Fig. 7G). SREBP-2 binding to these SREs was not detected (not shown).

An electrophoretic mobility shift assay was used to identify the binding site(s) for SREBP-1c. Only a mobility shift of the SRE1 probe was observed using nuclear extracts (NE) from HepG2 cells, when compared to SRE2 (Fig. 8A, lane 2 vs. 4). Binding was abolished when SRE1 was mutated (Fig. 8A, lane 2 vs. 3). A 20-fold cold competitor SRE1 probe also abolished binding (Fig. 8B, lane 2 vs. 3). In order to confirm that the protein-DNA complex formation was due to a SREBP-1c-SRE1 interaction, a gel-super shift assay was performed using anti-SREBP-1c polyclonal antibodies. Addition of this antibody to the nuclear extract resulted in a super shift in the protein-DNA complex (Fig. 8B, lane 2 vs. 3). Thus, SREBP-1c binds to SRE1 in the miR-185 promoter in order to regulate its expression in response to cholesterol level.

**miR-185 expression is induced by LXR activation and insulin treatment.**

LXR activates *SREBP-1c* expression in the presence of oxidized cholesterol. To determine if miR-185 expression was regulated by activation of LXR and induction of SREBP-1c level, HepG2 cells were treated with HPCD to remove cholesterol. They were then incubated with 25-HC, and the expressions of several LXR regulated genes were determined. *ABCA1*, *ABCG1*, and *SREBP-1c* expressions were all up regulated upon LXR activation when compared to control cells (Fig. 9). More importantly, miR-185 expression was induced ~9-fold, while *SREBP-2* expression was reduced by nearly 75% (Fig, 9). These results suggest that *SREBP-2* expression is regulated by increases in miR-185 level, brought about by LXR activation and subsequent induction of SREBP-1c transcription factor activity.
Insulin is a major activator of SREBP-1c in the liver (53, 54). To further validate that miR-185 expression was regulated by SREBP-1c, HepG2 cells were treated with 100 nM insulin, and SREBP-1c, miR-185, and SREBP-2 expression levels were determined. Upon insulin treatment, a decrease in full-length SREBP-1c (SREBP-1C (FL)) was observed with a concomitant increase in mature SREBP-1 (SREBP-1C (M)), indicating that insulin treatment did activate SREBP-1c (Fig. 10A & B). SREBP-1c mRNA level increased 2-fold upon insulin treatment comparing to control cells (Fig. 10C). miR-185 expression was also significantly increased in insulin treated cells, while SREBP-2 expression decreased (Fig. 10C). Thus, activation of SREBP-1c by insulin treatment caused an increase in miR-185 level, which in turn resulted in decreased SREBP-2 expression.

An increase in miR-185 expression level correlates with high blood cholesterol and reduced SREBP-2 protein in mice fed a high fat diet.

To explore the in vivo relevance of miR-185-dependent regulation of SREBP-2 expression, miR-185 expression, cholesterol and SREBP-2 levels, were determined in mice fed a high fat atherogenic diet (21% fat) or a normal fat diet (7% fat). Mice were fed each diet for 16 weeks, and blood and organs were harvested at weeks 4, 8, 12, 16. In mice fed a high fat diet, there was a time-dependent increase in blood cholesterol level, while control animals fed a normal diet did not show this increase (Fig 11A). The level of SREBP-1c and miR-185 also showed a time-dependent increase (Fig. 11B & C), while SREBP-2 expression and protein level decreased in mouse liver (Fig. 11D & E). Densitometry analysis of the western blot verified that SREBP-2 levels decreased in a time-dependent manner (Fig. 11F).
To further substantiate these results, mice were injected with miR-185 lentivirus, and miR-185, SREBP-2, LDLR, PCSK9, and HMGCR expressions levels were determined in liver at 12 weeks. miR-185 expression was up regulated in miR-185 lentiviral injected mice (~5-fold), when compared to mice injected with control virus (Fig. 12A, control vs. mir-185 O/E). In contrast, SREBP-2 expression decreased by 70% (Fig. 12B, control vs. mir-185 O/E). LDLR, PCSK9, and HMGCR expressions were also significantly decreased (Fig. 12C, control vs. mir-185 O/E).

**Human miR-185 expression is elevated in individuals with high cholesterol.**

Finally, miR-185 expression was determined in individuals with high cholesterol. Control patients had an average miR-185 expression ratio of ~10:1 (miR-185/RNU-6), while individuals with high cholesterol had ~50:1 ratio (Figure 13).

**DISCUSSION**

SREBP-2 acts as a master switch regulating the transcription of an array of genes that are critical for maintaining intracellular cholesterol homeostasis (8, 13). A better knowledge of the molecular mechanisms mediating SREBP-2 expression and function will help further the understanding of the complex systems in place to modulate cholesterol metabolism. Our findings have uncovered a novel mechanism by which SREBP-2 expression is post-transcriptionally repressed by miR-185. By decreasing SREBP-2 expression level, miR-185 negatively regulated SREBP-2-dependent gene expression, resulting in decreased expression of
several genes required for de novo cholesterol biosynthesis, and reduced LDLR protein and LDL uptake. Strikingly, miR-185 expression was regulated by SREBP-1c, thus setting up a possible cholesterol-responsive feedback loop. in vivo high fat feeding studies showed that high-fat fed mice had 1) an elevated expression of miR-185, and 2) reduced SREBP-2 protein. Moreover, mice overexpressing miR-185 had reduced SREBP-2 expression level and a reduction in the expressions of several SREBP-2-dependent genes. These results strongly suggest there is a novel miR-185 regulatory axis for the post-transcriptional regulation of SREBP-2 expression, whose activity responds to, and is regulated by, cholesterol level.

It is well established that MiRs play an important role in the initiation and/or progression of cardiometabolic disease (27, 35, 36, 55, 56). miR-122 was the first reported miR associated with the regulation of cholesterol homeostasis in liver (27, 35, 36, 57). The exact target genes regulated by miR-122, and the mechanism by which miR-122 regulates cholesterol level, remains to be fully elucidated. Another extensively studied group of miRs are the miR-33a/b family, which are found within the introns of SREBP-1 and SREBP-2. MiR-33a/b have several targets that include the ATP binding-cassette (ABC) A1 cholesterol efflux pump, inhibiting its expression, which results in increased intracellular cholesterol concentration under conditions of need (58, 59). Interestingly, miR-33a/b negatively regulate AMPK (35), a kinase that has been shown to phosphorylate and inhibit SREBP-2 activity (60). Thus, miR-33a/b expression would presumably up regulate SREBP-2 and cholesterol biosynthesis. In our case, miR-185 was up regulated through regulation by SREBP-1c. It targeted SREBP-2, shutting it down, likely ensuring that SREBP-2 did not increase cholesterol to a toxic level. Interestingly, miR-185 level was also up regulated in mice fed a high fat diet, and in human sera from patients with high cholesterol. Thus, it seems that in vivo, miR-185 regulates SREBP-2 activity when cholesterol
level is high in order to precisely fine-tune the level of this lipid. There are two other miRs, miR-4644 and miR-4306, that are proposed to target the same 3'UTR sequence as miR-185. A scan of their promoter sequences did not show any SREBP-1c binding sites. Thus, if they do regulate \textit{SREBP-2} expression, it is likely independent of SREBP-1c regulation.

The removal of cholesterol by MCD resulted in an acute activation of \textit{SREBP-2} and sterol gene expression, which was quickly blunted by overexpression of miR-185, suggesting that miR-185 regulation of \textit{SREBP-2} transcription may be fast and adaptable. Thus, tight transcriptional regulation of miR-185 expression is likely necessary under conditions where cholesterol level changes are drastic, such as times of fasting or after a high fat meal. \textit{SREBP-1c} expression is not regulated by cholesterol level to any measurable extent, thus its constitutive presence would ensure a direct rapid regulation of miR-185 expression. As SREBP-1c function decreases, so should miR-185 expression, resulting in a fine-tuning in cholesterol level by the tuning on and off of \textit{SREBP-2} function. The loss of SREBP-1c function in this scenario presumes that there is some type of modification, either changes in expression, or some type of post-translational modification. Thus, the three components in this loop need to be regulated by the same sensing mechanism. This same scenario is seen with miR-33a/b, as the \textit{SREBP-2} promoter regulates miR-33a/b expression (59). By inhibiting ABCA1 function and cholesterol efflux, miR-33a/b expression should be turned off, as should \textit{SREBP-2} expression, as the cholesterol level rises. It has been shown that nuclear receptors can directly bind to miRs promoters and control their expression.

Why would miR-185 be upregulated by a transcription factor (SREBP-1c) involved in fatty acid synthesis, as miR-185 inhibits \textit{SREBP-2} expression, thus reducing \textit{de novo} cholesterol biosynthesis and LDL uptake? \textit{SREBP-1c} expression is activated during a number of metabolic...
states (61-66), insulin (53, 54), and through LXRα activation by oxysterols (67, 68). This latter activation is believed to be required for the production of the fatty acids needed for cholesterol ester synthesis (69). Our results indicated that miR-185 expression was induced through LXR activation and resulting SREBP-1c expression. Based on this data, it is possible a SREBP-1c/miR-185 feedback loop exists in order to ensure that the ratio of free cholesterol/cholesterol esters is maintained. Inhibition of SREBP-2 function by miR-185 may result in a decrease in cholesterol level, allowing for the conversion of the remaining intracellular pool to non-toxic cholesterol esters. As the level of free fatty acids increase, SREBP-1c expression would decrease, miR-185 expression would be blunted, and SREBP-2 expression would increase. This would allow for the synthesis of the cholesterol needed to reduce the free fatty acid pool through esterification. It is well known that the expression of SREBP-1c is negatively regulated by an increase in polyunsaturated fatty acids (PUFA) (70-74). Interestingly, ACAT activity is elevated by PUFAs, thus setting up the use of fatty acids as substrates for cholesterol ester biosynthesis at a time when SREBP-1c activity is being reduced (75, 76). We point out that the hypothesis described is speculative and requires further studies to validate the model.

Our findings showed that SREBP-1c transcriptionally activated miR-185 expression, through binding to a specific SRE within the promoter region of miR-185, resulting in the suppression of SREBP-2-dependent events. Moreover, the knockdown of SREBP-1c actually resulted in increased SREBP-2 expression and protein. Thus, SREBP-1c inversely regulated SREBP-2 expression. Our finding may help to explain why SREBP-1c−/− mice show elevated SREBP-2 expression level in the liver (77, 78). Quite possibly, a decrease in miR-185 expression due to reduced SREBP-1c expression results in elevated SREBP-2 expression, acting as a compensatory mechanism. However, this mechanism may be toxic, as SREBP-1c−/− mice have a
3-fold increase in cholesterol biosynthesis in the liver, and a 50% increase in hepatic cholesterol level (77). Interestingly, the regulation of miR-185 expression was specific to SREBP-1c, as siRNA against SREBP-2 had no effect (M. Yang, W. Liu, and JT Nickels, unpublished data). SREBP-1a was not studied, as its expression level is extremely low in liver cells compared to SREBP-1c.

Although SR-BI is targeted by miR-185, how it decreases SR-BI level and affects cholesterol homeostasis still needs to be elucidated through further in vivo studies. As SR-BI was found to remove CE from circulating HDL, it was thought to regulate HDL-mediated RCT by taking up circulating HDL. Although SR-BI knock-out mice have increased plasma HDL, reduced hepatic cholesterol uptake, and decreased biliary cholesterol secretion (79), there are reports demonstrating SR-BI knock-out alone is not sufficient to induce an atherosclerotic phenotype in mice (80). It is likely that there is a compensatory SR-BI-independent mechanism for HDL uptake (79). in vivo studies looking at how miR-185 functions to regulate SR-B1 and how this regulation affects cholesterol homeostasis are necessary to provide more insight concerning miR-185 function and RCT.

Whether overexpressing miR-185 to inhibit SREBP-2 activity represents a feasible therapeutic for treating cholesterol-related diseases awaits further long-term in vivo mouse studies testing its efficacy in modulating SREBP-2 expression and lipid levels. It must be kept in mind that negatively regulating SREBP-2 may reduce LDLR expression, possibly leading to increased LDL and free fatty acid levels. However, it would also reduce PCSK9 expression. PCSK9 is involved in LDLR degradation. The loss of PCSK9 function may stabilize LDLR, minimizing the potential deleterious effects caused by loss of LDLR expression by SREBP-2. The idea of targeting SREBP-2 as a means to help treat hypercholesterolemia is beginning to
gain traction (81). Recently, Moon et al., (82) showed that siSCAP-treated mice showed reduced SREBP-2 expression, but maintained steady-state LDLR level, most likely due to a reduction in PCSK9 expression. Moreover, SREBP small molecule screening has identified, betulin, which enhances the SCAP-INSIG interaction (83). When administered to mice, betulin reduced hyperlipidemia and insulin resistance, while also decreasing atherosclerotic plaque formation (83). Thus, targeting SREBP for small molecule therapy warrants exploration.

Acknowledgements

We acknowledge and appreciate the financial support of the Genesis Biotechnology Group. We are grateful for the many discussions with Drs. Martin Adelson, Eli Mordechai, and Sui Lo, as well as, Mark Lieberman, Esq. and Christina Segro, Esq.. We thank Dr. Jason Trama for his input. We appreciate our colleagues at the Institute of Metabolic Disorders for their willingness to help with all aspects of the work.

REFERENCES


**Figure Legends**

**Figure 1.** miR-185 expression is decreased under conditions of cholesterol depletion. HepG2 cells were grown in the absence or presence of MCD and miR-185 expression was determined by qRT-PCR. (A) qRT-PCR was used to quantitate SREBP-2 mRNA and miR-185 level in HepG2 cells treated with or without MCD (10min). GAPDH and RNU6-2 were used as internal controls for mRNA and miR, respectively, (B) SREBP-2 protein level was examined by western blotting using whole cell lysate from HepG2 cells treated with or without MCD (10min). Actin served as a loading control. FL, full-length SREBP-2; M, mature form of SREBP-2.

**Figure 2.** miR-185 MREs in the SREBP-2 mRNA 3’UTR. A, A schematic of the SREBP-2 3’UTR region, and predicted miR-185 MREs. B, SREBP-2 3’UTR mutant reporter plasmid was generated by site-directed mutagenesis of the four potential miR-185 MREs (C to G).

**Figure 3.** miR-185 down-regulates SREBP-2 mRNA and protein level by binding to MREs within SREBP-2 mRNA 3’UTR. A, Luciferase activity was quantitated in 293T cells transfected with a control luciferase reporter plasmid (*Con Luc*), SREBP-2 3’UTR containing reporter plasmid (3’ *UTR*), SREBP-2 3’UTR mutant (3’ *UTR M*), and pre-miR-185 (*miR185*) or control pre-miR (*Con miR*). Luciferase activity was measured in 293T cells as described in the methods...
section. Bar graphs represent mean ± s.e.m from three independent experiments. B, The fold change in SREBP-2 mRNA was measured by qRT-PCR in pre-miR-185, Antagamer-miR-185 (100nM), or control miR transfected HepG2 cells. Bar graphs represent mean ± s.e.m from three independent experiments. C, SREBP-2 mRNA was measured by qRT-PCR in pre-miR-185 or control miR transfected THLE-2 cells. Values were normalized to the level of GAPDH. Bar graphs represent mean ± s.e.m from three independent experiments. D, SREBP-2 protein level was determined using whole cell lysates by western analysis in miR-185 over-expressing and control miR transfected HepG2 cells. β-actin was used as a loading control. FL, full-length SREBP-2; M, mature form of SREBP-2.

Figure 4. miR-185 regulates SREBP-2-dependent gene expression. (A, B & C). qRT-PCR was used to quantitate FDFT1, LDLR, and HMGCR mRNA levels in HepG2 cells transfected with pre-miR-185 or control pre-miR. Cells were treated with or without MCD (10 min) 48 hr post transfection. GAPDH was used as internal control. Bar graphs represent mean ± s.e.m from three independent experiments.

Figure 5. The loss of LDLR impairs LDL uptake in miR-185 over-expressing HepG2 cells. A, LDLR protein level was determined by western analysis in HepG2 cells transfected with control pre-miR or pre-miR-185 using whole cell lysates. β-actin was used as loading control. B, densitometry analysis of LDLR protein normalized to actin level. Bar graphs represent ±s.e.m from three independent experiments. C, Cell surface LDLR was determined by immunocytochemistry in HepG2 cells transfected with control pre-miR or pre-miR-185. DAPI was used as counter stain. Uptake of LDL by HepG2 cells were observed by incubating control
pre-miR or pre-miR-185 transfected HepG2 cells with LDL-BODIPY. DAPI was used to counter stain for nuclei. A Leica DMI6000 fluorescent microscope was used to visualize staining.

Figure 6. miR-185 over-expression decreases HMGCR activity in HepG2 cells. HMGCR activity was determined by normalizing isolated [14C]-mevalonolactone to the internal control, [3H]-mevalonolactone. Relative activity was calculated by normalizing HMGCR activity in the miR-185 over-expressing, MCD treated control cells (30 min), and MCD treated miR-185 over-expressing HepG2 cells, to control HepG2 cells. Graph represents mean ± s.e.m. of three independent experiments.

Figure 7. SREBP-1c down-regulates SREBP-2 via miR-185 up-regulation. A, Putative SREs within the promoter region of miR-185 transcript. B, Full Length (FL) and N-terminal (M) SREBP-1c protein expression was determined from whole cell lysates by western analysis in HepG2 cells transfected with SREBP-1c siRNA and control siRNA. β-actin was used as a loading control. (C & D) SREBP-2 mRNA expression (C) and mature SREBP-2 protein level (D) were determined in HepG2 cells transfected with SREBP-1c siRNA or control siRNA. GAPDH was used as internal control in qRT-PCR, and β-actin was used as a loading control for western blot analysis. Expression levels were quantitated by qRT-PCR. RNU6-2 was used as internal control. (E & F) Relative expression of mature miR-185 in SREBP-1c siRNA transfected HepG2 (E) and THLE2 cells (F) compared to control siRNA transfected cells. Expression levels were quantitated by qRT-PCR. RNU6-2 was used as internal control. G, ChIP assay was used to determine the extent of binding of SREBP-1C-c to the miR-185 promoter region. 10% of the cell extract was used as an input. Rabbit IgG was used as a negative control.
Primers detecting 50bp to 500bp upstream of the miR-185 TSS were used for PCR. *FL, full-length SREP-1c; M, mature form of SREBP-1c.*

**Figure 8.** SREBP-1c binds to SRE1 within the miR-185 promoter. An EMSA assay was used to determine SREBP-1c SRE binding within the miR-185 promoter. A, HepG2 nuclear extract was incubated in the presence of a WT SRE1 probe (lane 2), mutant SRE1 probe (sre1, lane 3), WT SRE2 probe (lane 4), and a mutant SRE2 probe (sre2, lane 5). B, HepG2 nuclear extract was incubated in the presence of a WT SRE1 probe (lane 2), WT SRE1 cold competitor (lane 3), SREBP-1c antibodies (lane 4), and GAPDH antibodies (lane 5). Free probe (WT biotin labeled SRE1 probe in nuclear extract) was used as control (lane 1). GAPDH antibody was used as negative control for non-specific antibody interaction. ** represents a supershift corresponding to decreased probe migration.

**Figure 9.** LXR activation results in an induction of miR-185 expression, while decreasing SREBP-2 expression. HepG2 cells were grown in serum free medium in the absence and presence of 2µg/ml 25-HC for 6 hr post cholesterol removal by 1% HPCD for 1hr at 37ºC. SREBP-1c, ABCA1, ABCG1, SREBP-2, and miR-185 expression levels were determined by qRT-PCR. GAPDH and RNU6-2 were used as internal controls for mRNA expression and miR-185 expression, respectively. The values are the average of three independent experiments.

**Figure 10.** Insulin treatment causes an induction of both SREBP-1c mRNA and miR-185 expression. HepG2 cells were grown in serum free medium with or without 100nM insulin overnight at 37ºC. A, SREBP-1c protein level was examined from whole cell lysates by western
blotting. GAPDH was used as internal control. B, Densitometry was used to normalize both full length and mature form of SREBP-1c protein levels to GAPDH. C, SREBP-1c, SREBP-2 and miR-185 expression level were determined by qRT-PCR (C). GAPDH and RNU6-2 were used as internal controls for mRNA expression and miR-185 expression, respectively. The values are an average of three independent experiments. FL, full-length SREBP-1c; M, mature SREBP-1c.

Figure 11. Mice fed a high fat diet express a higher level of miR-185. (A, B, C, & D) mice fed normal (7% fat) or atherogenic (21%) diets were sacrificed at weeks 4, 8, 12, and 16. Blood was drawn and organs were harvested. A, cholesterol level in blood, B, relative SREBP-1c expression in liver, C, Relative miR-185 expression in liver, D, SREBP-2 expression, E, SREBP-2 mature protein level in liver, F, SREBP-2 densitometry measurement, were determined at the indicated weeks. Levels were compared to control cells grown in the absence of 25-HC, and the value obtained was set at 1. n = 3-5 mice.

Figure 12. Mice overexpressing miR-185 harbor defects in SREBP-2 signaling. 6-8 week old WT male B6 mice were injected with 5X10^9 pfu lentivirus with or without primary miR-185 sequences, via retro-orbital injection. Two weeks post injection, mice were sacrificed and liver tissue was collected for miR analysis. A, the expression levels of miR-185, B, SREBP-2, and C, PCSK9, LDLR and HMGCR were determined using qRT-PCR. n = 3-5 mice. RNU-6 was used as an internal control.
Figure 13. miR-185 expression is elevated in individuals with high cholesterol. Blood from control (control, n = 40) or individuals with high cholesterol (high cholesterol, n=46) were obtained and miR-185 expression was determined using qRT-PCR.
1. CTCT(C→G)TCT(C→G)CCC
2. TCT(C→G)T(C→G)T(C→G)CTG
3. ACT(C→G)T(C→G)T(C→G)CTT
4. ATT(C→G)T(C→G)T(C→G)CCT

diagram 2
**Figure 3**

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Relative Luciferase Units $\times 10^5$

$p < 0.03$

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Relative Expression, SREBP2

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Relative Expression, SREBP2

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**Figure 4**

(A) Relative Expression, HMGCR

(B) Relative Expression, LDLR

(C) Relative Expression, FDXR

* $p<0.01$
Relative activity, HMGCR

- Con
- Con + MCD
- miR-185
- miR-185 + MCD

Figure 6
A

SRE sites in the miR-185 promoter
-356 GCCTGGGTACTCCACCTGAGGTTATA -331 (SRE1)

-133 CTGGAGCTCTCAAGCCACCTGCCC -110 (SRE2)

B

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Relative Expression, SREBP2

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HepG2

Relative Expression, miR-185

F

Relative Expression, miR-185

G

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Figure 12

Relative Expression, SREBP-2

Control

miR-185 O/E

p<0.01

Relative Expression, miR-185

Control

miR-185 O/E

p<0.01

Relative Expression

LDLR

HMGCR

PCSK9

Control

miR-185 O/E

p<0.01

p<0.01

p<0.01