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Activation of LDL Receptor Expression by Small RNAs Complementary to a Noncoding Transcript that Overlaps the LDLR Promoter

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SUMMARY

Low-density lipoprotein receptor (LDLR) is a cell-surface receptor that plays a central role in regulating cholesterol levels. Increased levels of LDLR would lead to reduced cholesterol levels and contribute to strategies designed to treat hypercholesterolemia. We have previously shown that duplex RNAs complementary to transcription start sites can associate with noncoding transcripts and activate gene expression. Here we show that duplex RNAs complementary to the promoter of LDLR activate expression of LDLR and increase the display of LDLR on the surface of liver cells. Activation requires complementarity to the LDLR promoter and can be achieved by chemically modified duplex RNAs. Promoter-targeted duplex RNAs can overcome repression of LDLR expression by 25-hydroxycholesterol and do not interfere with activation of LDLR expression by lovastatin. These data demonstrate that small RNAs can activate LDLR expression and affect LDLR function.

INTRODUCTION

The ability of small RNAs to inhibit gene expression by targeting mRNA is well established (Siomi and Siomi, 2009). Several reports have also appeared suggesting that small RNAs complementary to gene promoters can also regulate gene expression. These antigene RNAs (agRNAs) (we use this terminology to distinguish them from RNAs complementary to mRNA) can either inhibit (Morris et al., 2004; Ting et al., 2005; Janowski et al., 2005; Janowski et al., 2006; Kim et al., 2006; Han et al., 2007; Janowski et al., 2007; Napoli et al., 2009; Hawkins et al., 2009; Watts et al., 2010b; Yue et al., 2010) or activate gene expression (Li et al., 2006; Janowski et al., 2007; Morris et al., 2008; Place et al., 2008; Huang et al., 2010; Watts et al., 2010b; Yue et al., 2010). Argonaute 2 (AGO2), a key protein involved in RNAi (Siomi and Siomi, 2009), has been reported to be required for gene activation (Li et al., 2006; Morris et al., 2008; Chu et al., 2010), and both AGO2 and a related protein, AGO1, have been implicated in transcriptional silencing (Janowski et al., 2006; Kim et al., 2006; Napoli et al., 2009; Chu et al., 2010). RNA-directed transcriptional gene silencing in human cells has been recently reviewed (Morris, 2009; Pastori et al., 2010).

Gene silencing by double-stranded RNAs complementary to mRNA has rapidly moved from being a laboratory tool to a drug development strategy with several ongoing clinical trials (Watts and Corey, 2010). Disease targets span a wide range including viral infections, asthma, macular degeneration, and cancer. Some RNA drugs are delivered locally by intraocular or inhaled administration, whereas others are delivered systemically. Activation by RNA would supplement RNA-mediated gene silencing and broaden the pool of genes susceptible to therapeutic regulation by nucleic acids.

Our goal in this study was to examine how RNA-mediated gene activation could be used to enhance expression of a therapeutically significant gene. We used the following criteria for choosing a gene target: (1) there should be experimental or clinical data showing that enhanced expression of the target gene leads to a potentially favorable therapeutic outcome; and (2) the target gene should be expressed in the liver, an organ demonstrated to be accessible using current technology for in vivo RNA delivery (Soutschek et al., 2004; Wolfrum et al., 2007).

We chose the LDL receptor (LDLR) as a target for agRNAs. LDLR is a cell-surface receptor responsible for internalization of plasma LDL-cholesterol (LDL-c) (Goldstein and Brown, 2009). LDLR is expressed in almost all tissues, but liver is an important organ for uptake of plasma LDL-c, ~70% of which is removed in liver. Enhanced expression of hepatic LDLR decreases the level of plasma LDL-c, providing a strategy for treatment of hypercholesterolemia.

Although statins, inhibitors for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, have been widely used for lowering levels of plasma LDL-c, the response to statin treatment for lowering LDL-c varies due to genetic differences or other factors in each patient (Chasman et al., 2004; Voora et al., 2008). Therefore developing methods to increase the amount of LDLR expression might broaden therapeutic options. Proprotein convertase subtilisin/kexin type 9 (PCSK9) is one promising target whose expression is correlated with reduced levels of LDLR (Horton et al., 2009). Antisense oligonucleotides (Graham et al., 2007; Gupta et al., 2010) or duplex siRNAs (Frank-Kamenestsky et al., 2008) that reduce PCSK9 expression have been

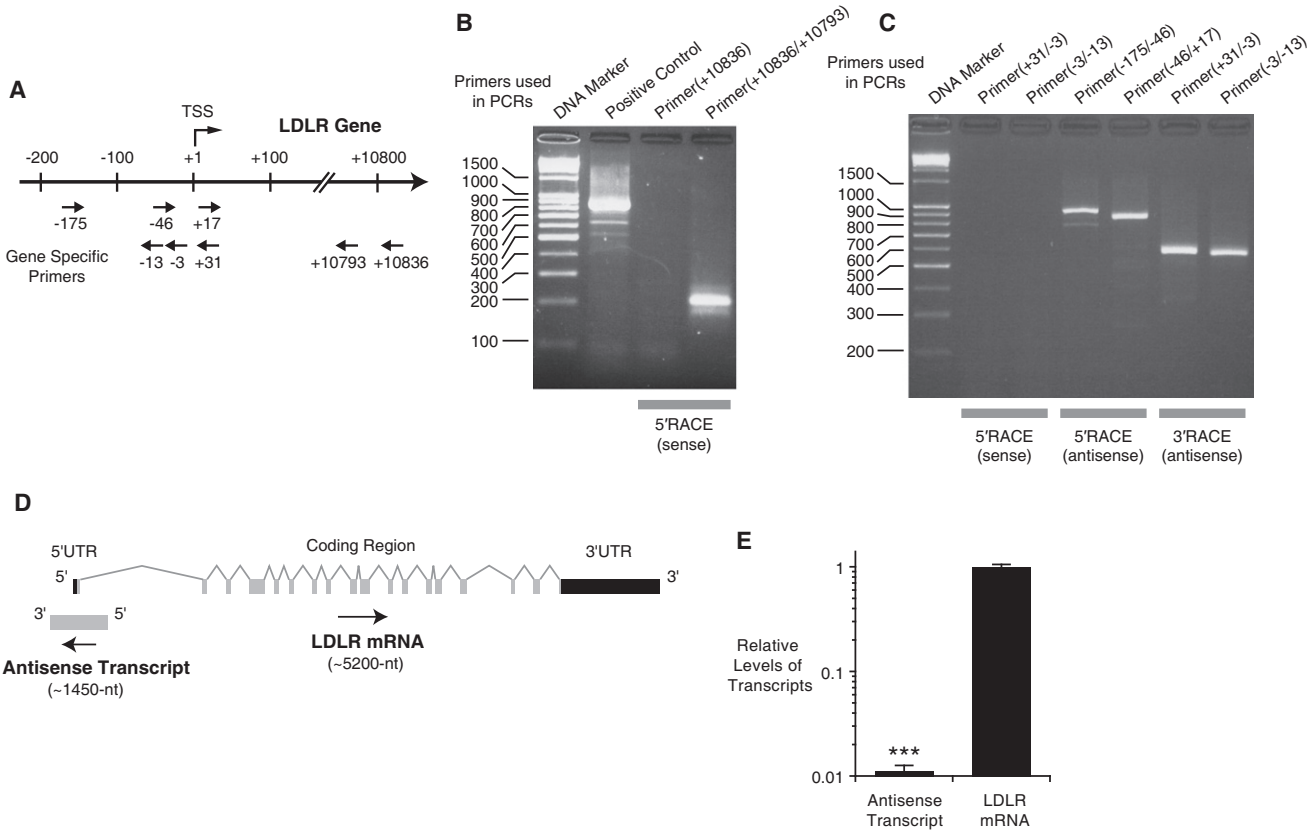

Figure 1. Transcripts at the LDLR Promoter

(A) Location of gene specific primers used in rapid amplification of cDNA ends (RACE).

(B) Analysis of RACE products defining the 5′ termini of LDLR mRNA. Primer + 10836 and primer + 10793 are gene specific primers complementary to exon 2 in LDLR mRNA. Positive control is a product (~900 base pairs) from a RACE using HeLa RT template and a control primer specific for β-actin cDNA.

(C) Analysis of 5′ and 3′ RACE products for sense or antisense noncoding transcripts. Nested PCRs were performed to increase specificity in amplification of target cDNAs. Gene specific primers used in the 1st/2nd(nested) PCRs are shown on top of each lane.

(D) Relative locations of LDLR mRNA and the antisense transcript.

(E) Relative expression levels of LDLR mRNA and the antisense transcript evaluated by qRT-PCR. ***$p < 0.001$ (unpaired t test). Error shown is standard deviation (SD). The transcription start sites and the 3′ ends identified by these RACE analyses are shown in Figures S1A–S1C. Results showing the connection between the 5′ and 3′ RACE products are presented in Figure S1D. The sequence of the antisense transcript is shown in Figure S1E.

shown to enhance LDLR expression by 2–3-fold and both types of nucleic acid are currently in preclinical development.

The LDLR gene is located on chromosome 19 (19p13.2) and its regulation has been well characterized (Südhof et al., 1987; Dawson et al., 1988; Smith et al., 1990; Briggs et al., 1993). The promoter contains three imperfect repeats and two TATA-like sequences. The transcription factor Sp1 binds to repeat 1 and 3, and sterol regulatory element binding proteins (SREBPs) bind to repeat 2. Transcriptional activity is controlled by a feedback mechanism through the processing of SREBPs and is negatively regulated by sterols. LDLR protein is glycosylated in the endoplasmatic reticulum (ER), generating LDLR precursor with an apparent molecular weight of 120 kDa. The precursor is subject to further glycosylation in the Golgi to be converted into mature LDLR with an apparent molecular weight of 160 kDa (Cummings et al., 1983). The mature LDLR is located on the cell surface and plays a role in uptake of LDL-c through endocytosis.

We find that LDLR expression can be enhanced by addition of small RNAs targeting the LDLR promoter. Levels of cell-surface LDLR increase and the enhancement of LDLR expression is similar to that achieved by the cholesterol-lowering drug lovastatin.

RESULTS

Characterization of Transcripts at the LDLR Promoter

Designing RNAs to target gene promoters requires an accurate identification of the transcription start site. We used rapid amplification of cDNA ends (RACE) to analyze start sites for LDLR mRNA in HepG2 cultured human liver cells (Figures 1A and 1B; see Table S1 available online). After sequencing 69 clones, we identified 14 transcription start sites for LDLR mRNA, and the +1 transcription start site was designated based on our 5′ RACE analysis and 5′ EST data from the database for transcription start sites (DBTSS: http://dbtss.hgc.jp/) (Figure S1A).

In previous studies of agRNA-mediated modulation of gene expression, we examined expression of progesterone receptor (PR). We observed that, rather than recognize chromosomal

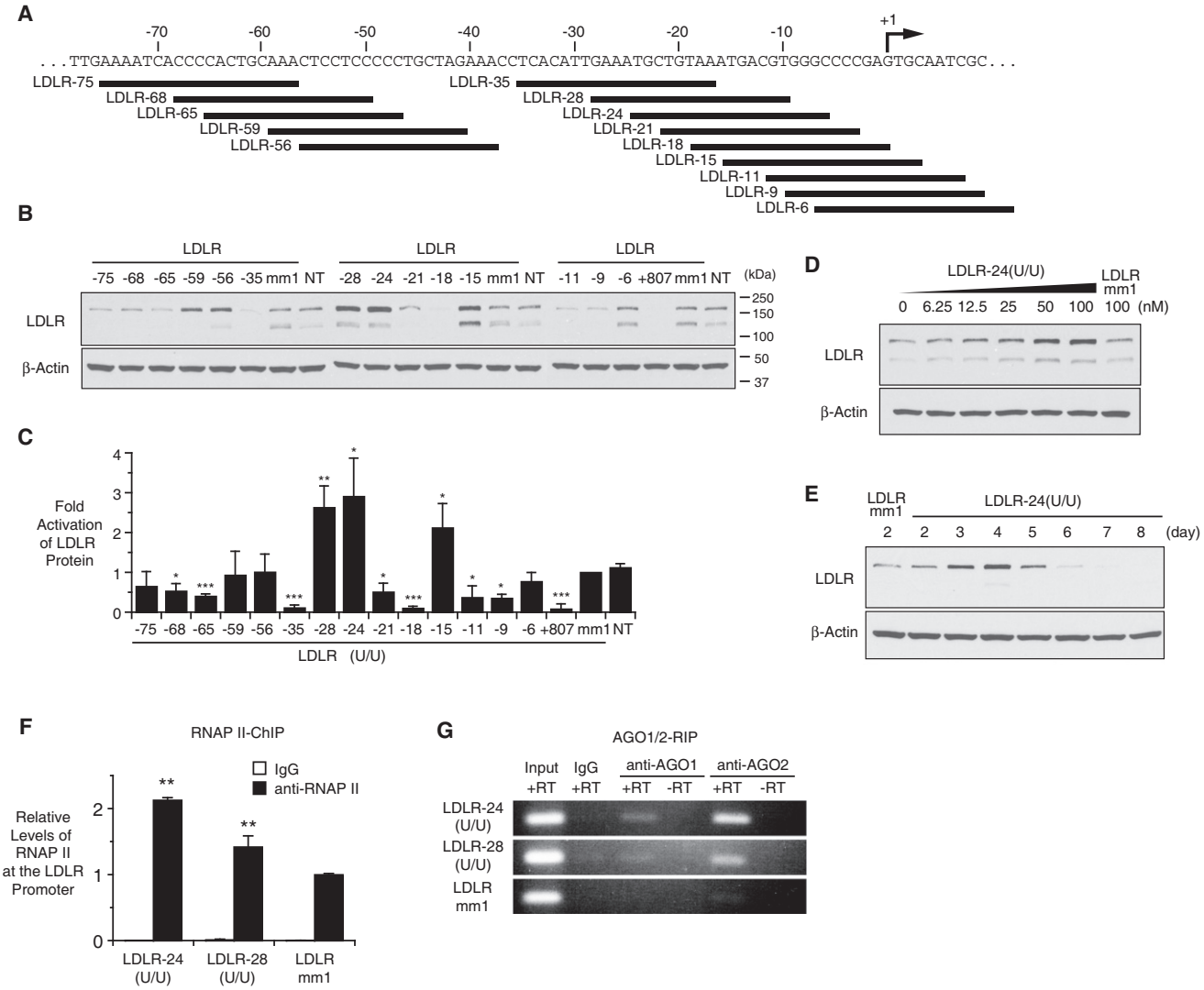

Figure 2. Identification and Characterization of agRNAs that Activate LDLR Expression

(A) Location of target sites for agRNAs relative to the +1 transcription start site for LDLR.

(B) Western analysis showing the effects of varied agRNAs (50 nM) on expression of LDLR in HepG2 cells.

(C) Quantitation of results shown in (B) and independent replicates (n = 4). Statistical significance relative to mismatch control LDLRmm1 was tested by paired t test. *p < 0.05; **p < 0.01; ***p < 0.001.

(D) Western blots showing a dose response for LDLR-24(U/U).

(E) Western blots showing a time course of LDLR expression after treatment with LDLR-24(U/U) (50 nM).

(F) ChIP of RNAP II using an anti-RNAP II antibody after treatment with activating agRNAs or mismatch control (50 nM). n = 3. Data were analyzed using Dunnett's test. **p < 0.01 relative to mismatch control LDLRmm1.

(G) RIP of AGO1 or AGO2 using an anti-AGO1 or anti-AGO2 antibody after treatment with activating agRNAs or mismatch control (50 nM). Error shown is SD. See also Figure S2 and Figure S5.

DNA, agRNAs recognize noncoding transcripts that overlap the PR gene promoter (Schwartz et al., 2008). The noncoding transcript at the PR promoter was an antisense transcript synthesized in a direction opposite to that of PR mRNA.

To investigate whether noncoding transcripts are expressed in the LDLR promoter, we performed 5′ and 3′ RACE using LDLR promoter-specific primers. We discovered a ~1450-nt antisense transcript that overlaps the LDLR promoter, initiating at ~+880 and terminating at ~−570 (Figures 1C and 1D; Figures S1B–S1E). This transcript is polyadenylated, unspliced, and expressed at levels ~90-fold below LDLR mRNA (Figure 1E). We did not detect sense transcripts overlapping the LDLR promoter, making the antisense transcript the most plausible target for anti-LDLR agRNAs.

Design of agRNAs

The agRNAs used in these studies were 19-base pair RNA duplexes with 2-base deoxythymidine overhangs at the 3′ ends (Table S2). The agRNAs were designed to be complementary to sequences throughout the promoter for LDLR (Figure 2A). agRNA

nomenclature is defined by the most upstream base. For example, LDLR-24 would target bases −24 to −6 relative to the +1 transcription start site for LDLR. LDLR+807 is a siRNA complementary to LDLR mRNA. It represses LDLR expression through the standard post-transcriptional RNAi mechanism and we used it as a positive control for evaluating transfection efficiency. Mismatch-containing dsRNAs LDLRmm1 and LDLRmm2 were designed based on the sequences of LDLR-24 and LDLR-28, respectively.

Activation of LDLR Expression by agRNAs

We transfected agRNAs into HepG2 cells and evaluated expression of LDLR protein by western blotting 4 days later. RNAs were transfected at 50 nM, a concentration chosen to combine maximal efficacy with minimal toxicity to cells. Western analysis revealed two immunoreactive bands due to the precursor and mature forms of LDLR described above. agRNAs LDLR-24, LDLR-28, and LDLR-15 increased LDLR protein levels by 2–3-fold (Figures 2B and 2C). Enhanced expression was dose-dependent and transient, reaching a maximum level 4 days after transfection (Figures 2D and 2E; Figures S2A and S2B). Activation of LDLR expression by LDLR-24 and LDLR-28 was characterized by potencies (EC_{50} values) of 26 and 16 nM, respectively (Figure S5).

Consistent with the gene activation at the level of protein, chromatin immunoprecipitation (ChIP) revealed 1.5- to 2-fold elevation of levels of RNA polymerase II (RNAP II) at the LDLR promoter (Figure 2F). Levels of the antisense transcript did not decrease after transfection of activating agRNAs (Figure S2C), suggesting that cleavage of the transcript by AGO2 does not appear to be a primary cause of the activation. We also monitored levels of H3K27 trimethylation (H3K27me3), which is a transcription-suppressive chromatin mark. Unlike our previous observation in activating agRNAs for PR (Yue et al., 2010), no significant changes were detected for the chromatin mark (Figure S2D). This might reflect that H3K27me3 is not a dominant regulatory factor for LDLR gene in HepG2 cells where basal expression level of the gene is relatively high.

To check cell specificity of LDLR activation by agRNAs, LDLR-24 and LDLR-28 were also tested in three other cell lines including HuH-7, fibroblast cells (GM04281), and SW480. We observed a similar effect of the oligomers on LDLR expression in the cell lines except for LDLR-24 in HuH-7 cells (Figure S2E).

When mismatch duplex RNAs were added, LDLR expression started to decrease 4–5 days after transfection (Figures S3H and S3I), probably due to a cellular response to the conditions where cholesterol is less required as cells become confluent. Thus the activation we observe runs counter to a natural tendency of LDLR expression to decrease over time.

There are four AGO proteins in mammalian cells (Siomi and Siomi, 2009). AGO2 is the "catalytic engine" that drives mRNA cleavage (Liu et al., 2004; Meister et al., 2004; Rand et al., 2004), whereas the roles of AGO1, AGO3, and AGO4 are less well known. We and others have previously reported that the action of promoter-targeted RNAs involves AGO1 or AGO2 (Li et al., 2006; Kim et al., 2006; Janowski et al., 2006; Morris et al., 2008; Napoli et al., 2009; Chu et al., 2010; Yue et al., 2010).

To determine whether AGO proteins might also be involved in agRNA-mediated activation of LDLR, we performed RNA immunoprecipitation (RIP) for AGO1 and AGO2 on addition of agRNAs. Using RIP we observed primary recruitment of AGO2 to the LDLR antisense transcript in cells treated with LDLR-24(U/U) or LDLR-28(U/U) (Figure 2G). Recruitment of AGO1 could also be detected but at lower levels. No PCR products were amplified in the samples without reverse transcription, suggesting that we were not detecting amplification of chromosomal DNA.

Testing Mismatch-Containing or Randomly Scrambled Oligomers

To evaluate whether sequence complementarity of agRNA to the LDLR promoter is required for activation, we tested another nine mismatch-containing or randomly scrambled RNA duplexes based on the sequence of LDLR-24 or LDLR-28 in addition to LDLRmm1 and LDLRmm2 (Figure 3A; Table S2). Mismatch-containing RNAs were designed to spread mismatches throughout the RNA or concentrate them in regions with potential seed sequences. Seed sequences contain positions 2–8 within the duplex RNA and complementarity between seed sequences and RNA targets is known to be an important determinant for successful RNAi.

With one exception, these control oligomers did not activate LDLR expression (Figure 3B; Figure S3). The exception was LDLRmm4 that contains three mismatches outside the seed sequence predicted for recognition of the antisense transcript. One explanation for activation by LDLRmm4 is that it preserves the potential to form necessary seed sequence interactions with the antisense transcript detected at the LDLR promoter. Consistent with this hypothesis, RIP experiments for the mismatch oligomers showed recruitment of AGO2 to the antisense transcript by active duplex LDLRmm4 that contained mismatches outside the seed sequence, but not by inactive duplex LDLRmm3 that contained mismatches disrupting the predicted seed sequence (Figure S3J).

Several RNA duplexes, notably LDLR-65, LDLR-35, and LDLR-18, appeared to reduce gene expression (Figures 2B and 2C). However, we observed that some of the scrambled oligomers induced nonsequence-specific silencing of LDLR gene (Figure S3), complicating interpretation of LDLR gene silencing by agRNAs. Because of the tendency toward nonspecific silencing and our focus on gene activation, we did not investigate gene silencing further.

Effect of Chemical Modifications on Activation of LDLR

Development of duplex RNAs as drugs will require chemical modifications to improve their stability, specificity, and potency (De Paula et al., 2007; Watts et al., 2008). Modifying siRNAs can reduce off-target effects resulting from the miRNA pathway (Jackson et al., 2006), the innate immune system (Judge and MacLachlan, 2008), or loading of the wrong strand (Bramsen et al., 2007).

To determine whether activation of LDLR would be compatible with chemical modifications commonly used during drug development, we tested introducing 2′-O-methyl or 2′-fluoro nucleotides into LDLR-24 or LDLR-28 (Figure 4A; Table S2). Each type of modified duplex is assigned two uppercase letters. The first letter describes the chemical modification of the sense strand, whereas the second letter describes modification of the

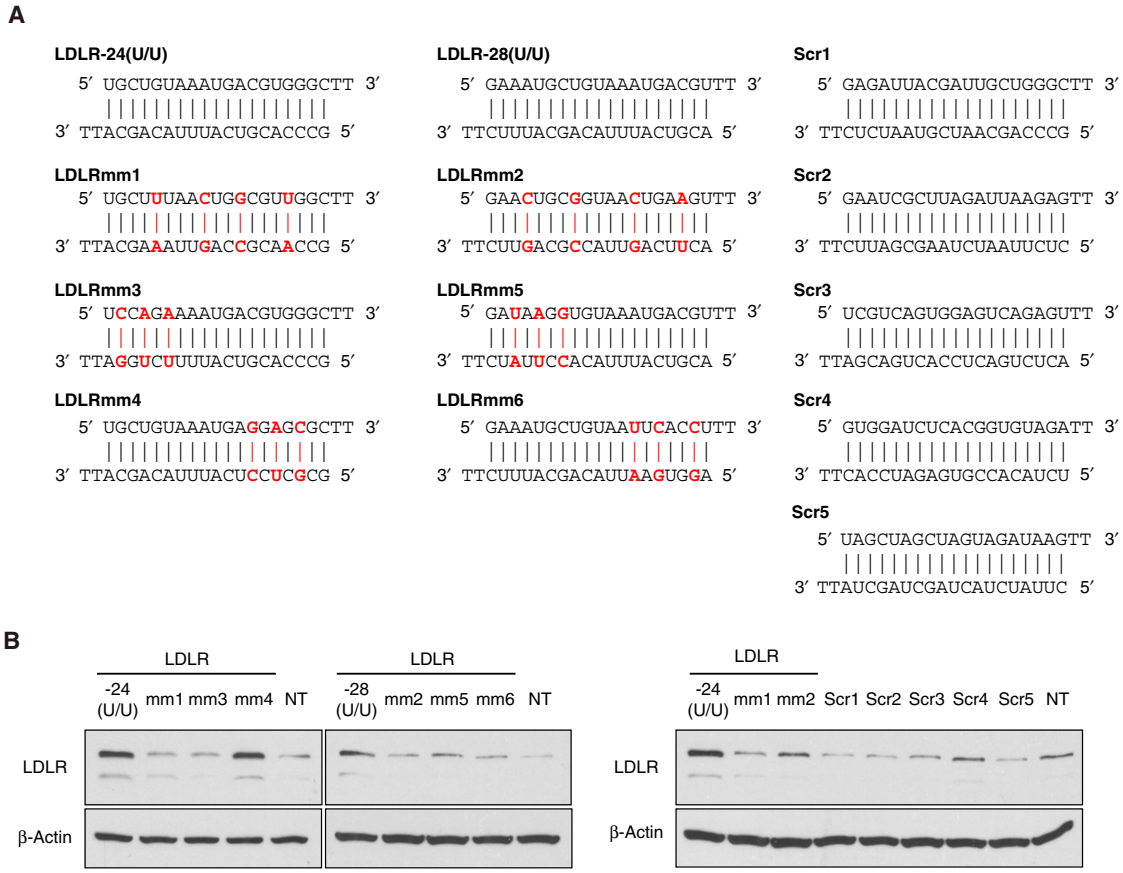

Figure 3. Effect of Mismatch-Containing Duplexes on Expression of LDLR

(A) The sequences of LDLR-24(U/U), LDLR-28(U/U), and corresponding mismatch oligomers. The upper strands are sense strands and the lower strands are antisense strands. Mismatch bases for LDLRmm1-6 are represented by red, bold face letters. Scrambled oligomers were generated by randomly scrambling the sequence of LDLR-24 or LDLR-28.

(B) Western analyses of LDLR expression for LDLR-24(U/U), LDLR-28(U/U), mismatch-containing oligomers LDLRmm1-6, and Scr1-5 (50 nM). NT indicates no treatment. Western blots are representative from at least three independent replicates for each experiment. See also Figure S3.

antisense strand. For example, U/F would have an unmodified sense strand and an antisense strand containing 2′-fluoro substitutions.

We observed activation of LDLR expression by chemically modified duplexes containing 2′-O-methyl or 2′-fluoro RNA (Figures 4B and 4C). Potencies (EC_{50} values) ranged from 4.1 to 38 nM (Figure S5). Maximal activation (A_{max}) was between 2.2- and 3.3-fold. For LDLR-24, activation was achieved with 2′-O-methyl RNA on the antisense strand or with 2′-fluoro RNA on the sense strand. When variants of LDLR-28 were tested, activation was observed regardless of whether the 2′-O-methyl or 2′-fluoro modifications were on the sense or antisense strand. The phenomenon that similar patterns of chemical modification have different effects on gene activation when applied to different sequences has been observed previously in chemically modified agRNAs that activate PR expression (Watts et al., 2010b).

The dependence of activation on the concentration of agRNA duplex was similar regardless of which modified agRNA was used (LDLR-24(U/O), LDLR-24(F/U), LDLR-28(U/O), or LDLR-28(F/U)) (Figure 4D; Figures S4A–S4C). Relative to unmodified LDLR-24(U/U) (Figure 2E), activation of LDLR by modified LDLR-24(U/O) persisted for a longer period, with elevated protein levels being observed until Day 6 after transfection (Figure 4E). These data demonstrate that agRNA-mediated activation of LDLR expression is compatible with chemical modifications commonly used during development of duplex RNA therapeutics.

Similar to our results for unmodified agRNAs, we did not observe any significant changes in the antisense transcript levels after treatment with chemically modified agRNAs (Figure S4D). In ChIP experiments for RNAP II, ~1.5-fold increase of RNAP II was observed at the LDLR promoter (Figure S4E). These results suggest that mechanism of the LDLR activation is conserved between unmodified and modified oligomers.

Upregulation of Cell-Surface LDLR

To examine whether agRNA-mediated activation of LDLR expression would lead to enhanced display of LDLR on the cell-surface and greater binding of LDL particles to the receptors, we performed LDL binding assay using 3,3′-dioctadecylindocarbocyanine labeled LDL (DiI-LDL). After treating cells with an activating agRNA or a mismatch control, the cells were

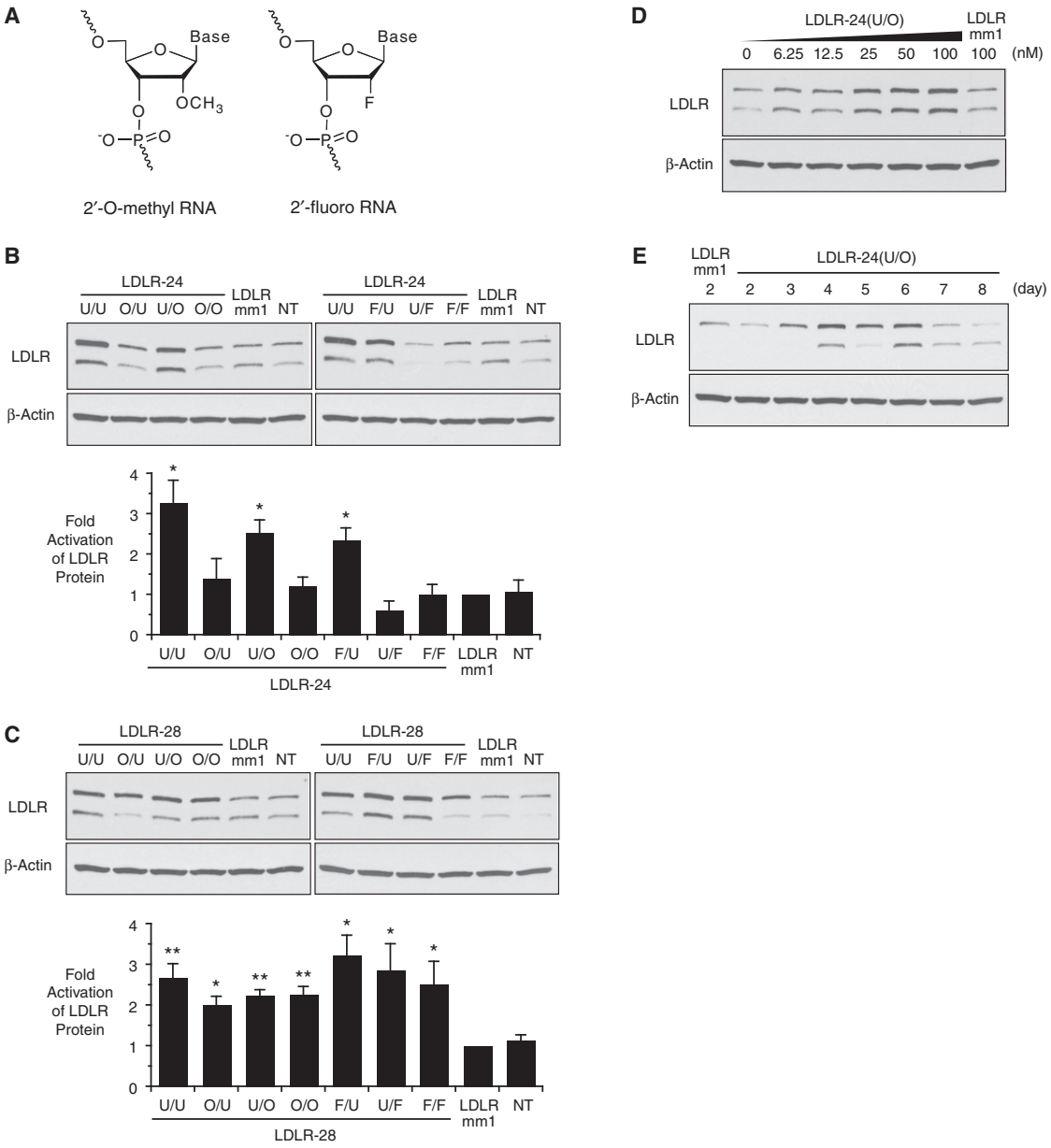

Figure 4. Effect of Chemical Modifications on RNA-Mediated Activation of LDLR

(A) Structures of 2′-O-methyl RNA and 2′-fluoro RNA.

(B) Effect of 2′-O-methyl and 2′-fluoro modifications on activation by LDLR-24 (50 nM). Representative western blots (top) and quantification of three independent replicates (bottom) are shown.

(C) Effect of 2′-O-methyl and 2′-fluoro modifications on activation by LDLR-28 (50 nM). Representative western blots (top) and quantification of three independent replicates (bottom) are shown.

(D) Western blots showing a dose response for LDLR-24(U/O).

(E) Western blots showing a time course profile of LDLR expression after treatment with LDLR-24(U/O) (50 nM) in HepG2 cells. Statistical significance relative to mismatch control LDLRmm1 was evaluated by paired t test. *$p < 0.05$; **$p < 0.01$. Error shown is SD. See also Figure S4 and Figure S5.

incubated with DiI-LDL and binding of DiI-LDL to the cell-surface was measured by fluorescence microscopy. We observed increased fluorescence in cells treated with DiI-LDL after addition of LDLR-24(U/U) relative to cells treated with the mismatch control LDLRmm1 (Figure 5A). Addition of unlabeled LDL quenched the fluorescence, indicating that the interaction is specific.

Binding of DiI-LDL to the cell-surface was quantified using flow cytometry. Cells treated with varying concentrations of activating agRNAs or a mismatch control were incubated with DiI-LDL and fluorescence from DiI-LDL bound to the cell-surface was measured. We observed enhanced fluorescence from DiI-LDL in LDLR-24(U/U)- or LDLR-28(U/U)-treated cells relative to LDLRmm1-treated cells in a dose-dependent manner (Figures 5B

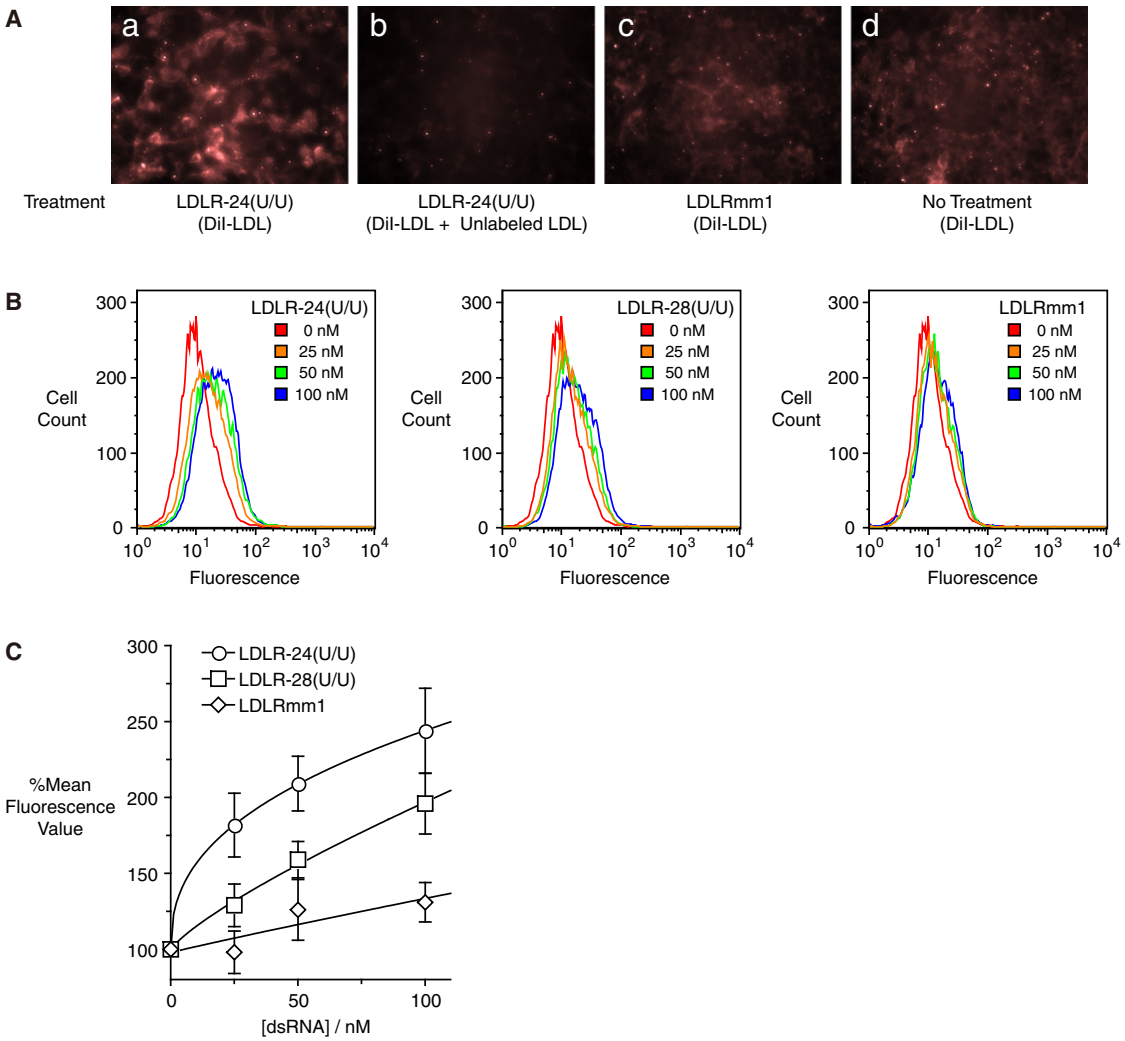

Figure 5. Binding of LDL to Cell Surface LDLR

(A) Fluorescent microscopy of HepG2 cells 4 days after transfection of LDLR-24(U/U) or LDLRmm1 (50 nM), or no treatment. Cells were treated with DiI-LDL (12 μg/ml) or a mixture of DiI-LDL (12 μg/ml) and unlabeled LDL (120 μg/ml) at 4°C for 2 hr.

(B) Flow cytometry showing DiI-LDL association. Varying concentrations of LDLR-24(U/U), LDLR-28(U/U), or LDLRmm1 were transfected into HepG2 cells. Four days after transfection, cells were treated with DiI-LDL (12 μg/ml) at 4°C for 2 hr and fluorescence from DiI-LDL bound to cells was measured by FACScan.

(C) Quantitation of cell surface-bound DiI-LDL after treatments shown in (B). Mean fluorescence value for no treatment sample was expressed as 100%. Error shown is standard error of the mean (SEM); n = 5.

and 5C). These results indicate that upregulation of LDLR by agRNAs led to enhanced trafficking of LDL particles to cell surface.

Effect of agRNAs on Expression of Interferon Responsive Genes

Some small RNAs can induce off-target effects through induction of the interferon response (Hornung et al., 2005; Birmingham et al., 2006). This potential activity is important for studies with LDLR because some cytokines have been reported to promote enhanced LDLR expression and increased LDL binding in cells (Stopeck et al., 1993; Ruan et al., 1998). To investigate involvement of interferon response to LDLR activation by agRNAs, we evaluated expression of interferon responsive genes by quantitative reverse transcription-PCR (qRT-PCR) after transfection of unmodified or modified agRNAs, LDLR-24(U/U), LDLR-24(U/O), LDLR-24(F/U), LDLR-28(U/U), LDLR-28(U/O), and LDLR-28(F/U). These agRNAs yielded only small changes for levels of interferon-responsive gene expression including OAS1, OAS2, MX1, IFITM1, and ISGF3γ (Figures 6A and 6B). Addition of polyinosinic-polycytidylic acid (poly I:C), a potent inducer of interferon response, substantially increases interferon responsive gene expression, but did not upregulate LDLR expression at any concentrations tested in HepG2 cells (Figures 6C and 6D). Taken together, these data suggest that gene activation by LDLR-24, LDLR-28, and their chemically modified variants is not due to induction of interferon-responsive genes.

Addition of agRNAs and 25-Hydroxycholesterol

The membrane-bound transcription factor SREBP binds to a sterol regulatory element within the LDLR promoter and

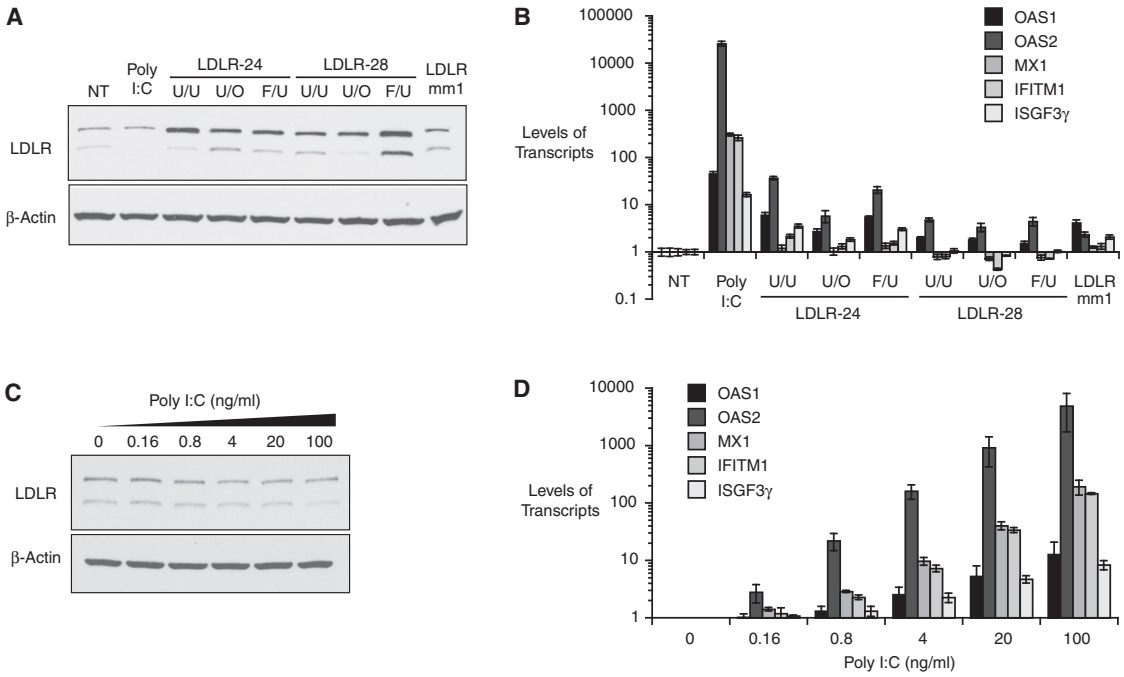

Figure 6. Effect of Treatment with Activating agRNAs or poly I:C on Expression of Interferon-Responsive Genes and LDLR

(A) Western analysis showing effect of activating agRNAs (50 nM) or poly I:C (100 ng/ml) on LDLR expression.

(B) qRT-PCR analysis showing effect of activating agRNAs or poly I:C on the expression of interferon responsive genes using cells examined in (A). n = 3.

(C) Western blots showing effect of poly I:C on LDLR expression.

(D) qRT-PCR analysis showing effect of poly I:C on the expression of interferon responsive genes using cells examined in (C). n = 3. Western blots are representative from three independent replicates. Error shown is SD.

triggers increased transcription of the LDLR gene (Brown and Goldstein, 1997). 25-Hydroxycholesterol represses LDLR expression by inhibiting the processing step that yields active NH_2-terminal fragments of SREBP (Adams et al., 2004). To determine whether addition of agRNAs might override this repression and permit enhanced LDLR expression, we added agRNA LDLR-24(U/U) or LDLR-28(U/U) in combination with 25-hydroxycholesterol.

We observed that LDLR-24(U/U) activated LDLR expression regardless of whether 25-hydroxycholesterol was present. Because treatment with 25-hydroxycholesterol lowers baseline LDLR expression, the relative activation by anti-LDLR agRNAs increased from 2–3-fold in cells grown under standard conditions to 4–9-fold (Figure 7A). This result has practical importance because, by suppressing basal expression, agRNA-mediated activation can be observed more clearly. Screening for activating agRNAs using cells treated to reduce basal levels of gene activation may be a useful strategy for more rapidly identifying the most promising agRNAs. Similar increases of LDLR expression were achieved using chemically modified agRNAs LDLR-24(U/O) and LDLR-28(F/U) in the presence of 25-hydroxycholesterol (Figure S6).

Addition of agRNAs and Lovastatin

Lovastatin is an HMG-CoA reductase inhibitor whose administration leads to increased levels of LDLR (Alberts, 1988). It is a United States Food and Drug Administration (FDA)-approved drug for lowering plasma LDL-c and comparing its activity with agRNAs offers a useful metric for evaluating the potential of agRNA-mediated modulation of LDLR expression. Addition of agRNA LDLR-24(U/U) or lovastatin alone led to an similar increase in expression of LDLR (Figure 7B). When we combined lovastatin and LDLR-24(U/U) in HepG2 cells, LDLR levels were significantly greater than when either agent was added individually, suggesting that the activities of lovastatin and anti-LDLR agRNAs are additive.

DISCUSSION

There are many strategies for using synthetic molecules to interfere with the function of a protein or block its expression. Small molecules can bind to protein targets and block their activity. Larger molecules like duplex RNAs or antisense oligonucleotides can bind to mRNA and reduce the levels of a target protein inside cells. Molecules that selectively enhance the activity of a target protein are more rare and their design poses a major challenge for research at the interface of chemistry and biology.

One promising strategy for enhancing gene expression is development of artificial transcription factors (Mapp and Ansari, 2007; Lee and Mapp, 2010). Several laboratories have reported compounds that consist of a DNA binding domain and a -transcriptional activation domain capable of recruiting transcription factors (Stanojevic and Young, 2002; Kwon et al., 2004; Liu et al., 2005; Xiao et al., 2007; Buhrlage et al., 2009). These

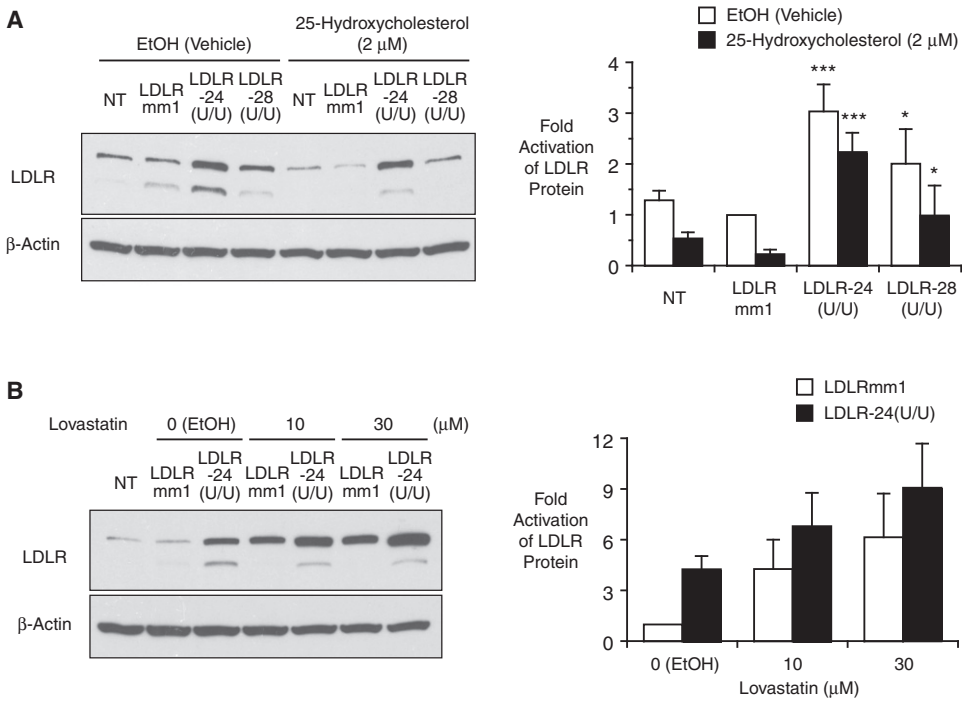

Figure 7. Combination Treatment of Activating agRNAs and 25-Hydroxycholesterol or Lovastatin

Fifty nanomolar duplex RNAs were used in these experiments.

(A) 25-Hydroxycholesterol (2 μM) or EtOH (vehicle) was added to cell culture media 2 days after transfection of activating agRNA LDLR-24(U/U), LDLR-28(U/U), or a mismatch oligomer LDLRmm1. Data shown are western blots of LDLR expression on day 4 (left) and quantitation of five independent replicates (right). Statistical significance was evaluated by paired t test. *$p < 0.05$; ***$p < 0.001$ relative to mismatch control LDLRmm1.

(B) Lovastatin (10 or 30 μM) or EtOH (vehicle) was added to cell culture media 2 days after transfection of activating agRNAs or a mismatch oligomer. Data shown are western blots of LDLR expression on day 4 (left) and quantitation of three independent replicates (right). Upregulation of LDLR expression by LDLR-24(U/U) or lovastatin was statistically significant (two-way ANOVA; $p < 0.01$). No significant interaction effects were detected between the two different treatments using agRNAs and lovastatin. NT indicates no treatment. Error shown is SD. See also Figure S6.

chemical approaches are simple and direct, but will require optimization of cellular uptake, DNA binding, and gene activation before activation at endogenous genes can be readily achieved inside cells.

Synthetic small duplex RNAs designed to recognize sequences at gene promoters provide an alternate strategy for controlling gene expression. One advantage of activating small RNAs is that several small RNAs are already being developed in the clinic for gene silencing. The chemical and pharmacological platform is, therefore, already well established. Activating RNA has the potential to significantly extend the reach of RNA to new targets for therapeutic development.

We find that duplex RNAs complementary to the LDLR promoter increase LDLR expression by 2–3-fold. This increase is comparable to the enhancement caused by treatment with lovastatin, a pharmacologically useful regulator of LDLR expression. Previously, Chen et al. (2008) have reported that promoter-targeted RNAs that yield RNA-mediated activation of p21 induced an antiproliferative effect in bladder cancer cells. These two reports suggest that RNA-mediated gene activation can be used to elevate expression of genes that are targets for therapeutic development.

We have previously shown that a promoter-associated antisense transcript is involved in activation of PR and changed abundance and/or localization of nuclear proteins such as hnRNP-k and HP1γ in the PR promoter (Schwartz et al., 2008). Activation of LDLR shares several similarities with activation of PR: (1) the antisense transcript is the most plausible target; (2) RNA Pol II is more abundant in the LDLR promoter after transfection of activating agRNAs; and (3) activating agRNAs recruit AGO proteins to the antisense transcript in the cell nucleus. Other labs have also reported similar recruitment of AGO to noncoding transcripts at gene promoters (Han et al., 2007; Morris et al., 2008; Hawkins et al., 2009; Napoli et al., 2009).

Data from our studies with agRNAs that target LDLR and PR suggest that agRNAs modulate gene expression by recruiting AGO2 protein to noncoding antisense transcripts at gene promoters (Schwartz et al., 2008; Yue et al., 2010; Janowski and Corey, 2010). This association occurs while the noncoding transcript remains associated with the promoter. Because the noncoding transcript is near promoter DNA, the AGO2/agRNA complex can interact with the transcription and other regulatory factors that normally control expression of PR or LDLR. A similar mechanism has been proposed to explain RNA-mediated epigenetic silencing in yeast (Grewal and Moazed, 2003).

The 2–3-fold increase in expression of LDLR protein, although large enough to be physiologically relevant, is small, making quantitative evaluation challenging. Relative to our previous experience activating PR, where 25–50-fold increases in protein levels were common, the smaller changes of LDLR expression

require more experimental replicates for validation. However, when basal levels of expression are reduced by addition of 25-hydroxycholesterol, the relative amount of activation increases and fewer replicates are required. Screening for RNA activators of other genes will be facilitated by assays using conditions that reduce the basal level of target gene expression.

SIGNIFICANCE

We describe activation of LDLR by promoter-targeted dsRNAs in cultured human liver cells. Our results show that gene activation can be achieved for a target protein known to be important for therapy of hypercholesterolemia and yield functional enhancement of the cell-surface LDLR in agRNA-treated cells. Chemically modified oligomers can also activate LDLR expression, suggesting that the approach of using activating agRNAs for therapeutic targets is compatible with in vivo applications. We identified several candidate agRNAs after screening a relatively small pool of promoter-targeted dsRNAs. Therapeutic use of activating agRNAs might provide an option for development of RNA-based drugs.

EXPERIMENTAL PROCEDURES

RACE Analysis

RACE was performed using the GeneRacer Kit (Invitrogen). cDNA samples from HepG2 cells were prepared according to the kit manufacturer's protocol. The 5′ or 3′ end of cDNA was amplified through two nested PCR steps using Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) and appropriate primer sets (Table S1). The thermal cycling condition of the first PCR was: 94°C for 2 min, followed by five cycles of 94°C for 30 s and 72°C for 1 min, five cycles of 94°C for 30 s and 70°C for 1 min, and 25 cycles of 94°C for 30 s, 66°C for 30 s, and 68°C for 1 min. The condition of the following nested PCR was: 94°C for 2 min, followed by 20 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 1 min. After gel purification, the PCR products were cloned into a pCR4-TOPO vector and sequenced (McDermott sequencing core, UT Southwestern).

qRT-PCR

Total RNA was extracted using TRIzol (Invitrogen). RNA samples were treated with DNase I (Worthington Biochemical) at 25°C for 10 min and reverse transcription was performed using High Capacity Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed on a 7500 real-time PCR system (Applied Biosystems) using iTaq SYBR Green Supermix (Bio-Rad). Primer sequences are described in Table S1. Standard curves for each primer set were made to evaluate primer efficiency in PCR amplification. qPCR data for comparing expression levels of LDLR mRNA and the antisense transcript were normalized by the difference in primer efficiency.

Cell Culture and Transfection

Unmodified, 2′-O-methyl, and 2′-fluoro RNAs with two 2′-deoxythymidine bases at the 3′ end were obtained from Integrated DNA Technologies or Alnylam Pharmaceuticals. HepG2 (American Type Culture Collection (ATCC)) and fibroblast cells (GM04281; Coriell) were cultured with Minimum Essential Medium Eagle (MEM; Sigma) supplemented with 10% FBS, 1% MEM nonessential amino acids (Sigma), and 1 mM sodium pyruvate (Sigma). HuH-7 (Japanese Collection of Research Bioresources) and SW480 cells (ATCC) were cultured with Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS and 1 mM sodium pyruvate. Cells were plated in 6-well plates at 120,000 (HepG2 and HuH-7), 60,000 (fibroblast), or 150,000 (SW480) cells/well 2 days before transfection. Duplex RNAs were transfected into cells using Lipofectamine RNAiMAX (Invitrogen). Cationic lipid (2.4 μl for 50 nM dsRNA) was added to OptiMEM (Invitrogen) containing oligonucleotides and the oligonucleotide-lipid mixture (250 μl) was incubated at room temperature for 20 min. OptiMEM (for HepG2 and fibroblast) or full media (for HuH-7 and SW480) was added to a final volume of 1.25 ml and the mixture was applied to cells. Media was exchanged 1 day later with fresh supplemented media (2 ml).

ChIP/RIP

HepG2 cells were seeded at 1,080,000 cells in 15 cm dishes 2 days before transfection for ChIP or RIP experiments. Two dishes were treated with activating agRNAs (LDLR-24[U/U] and LDLR-28[U/U]) or mismatch controls (LDLRmm1, LDLRmm3, and LDLRmm4) (50 nM). Four days after transfection, cells were crosslinked with 1% formaldehyde. Cells were recovered by scraping and nuclei were isolated using hypotonic lysis buffer (5 ml; 10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40). Nuclei were lysed in lysis buffer (1 ml; 1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], 1× Roche protease inhibitors cocktail, 40 U/mL RNasin Plus RNase Inhibitor [Promega]) and sonicated (2 pulses, 20% power, 20 s).

The cell lysate (100 μl) was incubated overnight with antibodies in immunoprecipitation buffer (1 ml; 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, and 1× Roche protease inhibitors cocktail, 40 U/ml RNasin Plus RNase Inhibitor). Monoclonal anti-RNAP II (2 μg; Millipore) and polyclonal anti-H3K27me3 (2 μg; Millipore) antibodies were used for ChIP experiments. Polyclonal anti-AGO1 (2 μg; Millipore) and polyclonal anti-AGO2 (2 μg; Millipore) antibodies were used for RIP experiments. Normal mouse IgG (2 μg; Millipore) or normal rabbit IgG (2 μg; Millipore) was used as a control. After the antibodies were recovered with 50 μl of Protein G Plus/Protein A Agarose Beads (Calbiochem), the beads were washed with 1 ml of low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), high salt (see low salt but with 500 mM NaCl), LiCl solution (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.1]), and TE buffer (pH 8.0). Protein was eluted twice with 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$, and 40 U/ml RNasin Plus RNase Inhibitor) for 15 min at room temperature. Crosslinking was reversed by adding NaCl to 200 mM and heating at 65°C for at least 2 hr. Protein was digested by incubating with Proteinase K (20 μg; Invitrogen) at 42°C for 50 min, followed by phenol extraction using an equal volume of phenol/chloroform/isoamyl alcohol. DNA/RNA in the aqueous layer was precipitated using 1/10 volume 3 M sodium acetate (pH 5.5), 2.2 volumes ethanol, and glycogen (40 μg; Sigma). For ChIP, the pellet was resuspended in 80 μl of nuclease-free water. qPCR was performed using iTaq SYBR Supermix and primers specific for the LDLR promoter (5′-CCTGCTAGA AACCTCACATTG-3′; 5′-GGATCACGACCTGCTGTGTC-3′). For RIP, the pellet was resuspended in 16 μl of nuclease-free water. After treating each sample with DNase I at 25°C for 10 min, reverse transcription reactions were performed only for input and +RT samples. qPCR was performed using iTaq SYBR Supermix and primers specific for the antisense transcript. PCR products were analyzed on 2.5% agarose gel and stained with ethidium bromide.

Analysis of LDLR Protein Expression

Cells were harvested 4 days after transfection for western blotting analysis. Cells were detached from plates using cell dissociation solution (Sigma) and lysed with lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT, and protease inhibitor [Calbiochem]). Protein concentrations were quantified with BCA assay kit (Thermo Scientific). SDS-PAGE was performed using 7.5% Tris-HCl gels (Bio-Rad). Gels were run at 100 V for 60 min. After gel electrophoresis, proteins were transferred to nitrocellulose membrane (Hybond-C-Extra; GE Healthcare) at 100 V for 2 hr. After blocking the membrane with 5% nonfat dry milk/TBST at room temperature for 1 hr, the membrane was incubated with primary antibody specific for LDLR or β-actin at the following dilution ratio: anti-LDLR antibody (ab52818; 1:10,000; Abcam), anti-β-actin antibody (1:20,000; Sigma). HRP-conjugated anti-rabbit (1:10,000; Jackson ImmunoResearch) or anti-mouse (1:20,000; Sigma) secondary antibody was used for visualizing proteins using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Protein bands were quantified using ImageJ software.

LDL Binding Assay

agRNAs (50 nM) were transfected in HepG2 cells as described above (Day 0). On day 4, cells were washed with cold PBS three times and then incubated with DiI-LDL (12 μg/ml; Invitrogen) or DiI-LDL (12 μg/ml) + unlabeled LDL (120 μg/ml; Invitrogen) in serum-free MEM at 4°C for 2 hr. After the incubation, cells were washed with cold PBS five times and then treated with 4% paraformaldehyde at room temperature for 25 min. After the fixation, cells were washed with PBS twice. Cells were observed using fluorescence microscopy (Zeiss Axiovert 200 M).

agRNAs (LDLR-24(U/U), LDLR-28(U/U), and LDLRmm1; 0, 25, 50, 100 nM) were also transfected into HepG2 cells for flow cytometry experiments. Four days after transfection, cells were harvested using cell dissociation solution and washed with 1 ml of PBS. After filtering cells using cell strainers (40 μm; BD Falcon), 250,000 cells in 250 μl of serum-free MEM were incubated with DiI-LDL (3 μg) at 4°C for 2 hr. Cells were collected by centrifugation (2500 rpm, 5 min) and then washed three times with 1 ml of PBS containing 0.5% BSA and 0.02% sodium azide. The fluorescence of cell-associated DiI-LDL was measured by FACScan (Becton Dickinson) with 10,000 cells per sample.

Analysis of Interferon Responsive Genes

mRNA levels of the interferon responsive genes including OAS1, OAS2, MX1, IFITM1, and ISGF3γ were measured by qRT-PCR. agRNAs (50 nM) and Poly I:C (0–100 ng/ml; Sigma) were transfected into HepG2 cells using the cationic lipid as described above. Three days after transfection, total RNAs from dsRNA-treated, poly I:C-treated, or untreated samples were isolated using TRIzol. The RNAs were treated with DNase I at 25°C for 10 min, followed by reverse transcription reaction at 37°C for 2 hr. qPCR was performed using iTaq SYBR Supermix and primers specific for the interferon responsive genes (Interferon Response Detection Kit; System Biosciences). LDLR protein levels on day 4 were also measured by western blot analysis.

Combination Treatment with Lovastatin and agRNAs

Inactive lovastatin (17 mg; Sigma) in the lactone form was converted into its active form as previously described (Morimoto et al., 2006). The stock solution (5 mM in 5% EtOH) was stored at −80°C until use. dsRNAs (50 nM) were transfected into HepG2 cells as described above (Day 0), and the media were exchanged one day later. Two days after transfection, lovastatin (10 or 30 μM) or 5% EtOH solution (vehicle) was added to each dsRNA-treated cell (final EtOH concentration: 0.03%). The cells were harvested on Day 4 for western blot analysis.

Combination Treatment with 25-Hydroxycholesterol and agRNAs

dsRNAs (50 nM) were transfected into HepG2 cells as described above (Day 0), and the media were exchanged one day later. Two days after transfection, 25-hydroxycholesterol (2 μM; Sigma) dissolved in EtOH or EtOH only (vehicle) was added to each dsRNA-treated cell (final EtOH concentration: 0.04%). The cells were harvested on Day 4 for western blot analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and two tables, and can be found with this article online at doi:10.1016/j.chembiol.2010.10.009.

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