RP1-13D10.2 Is a Novel Modulator of Statin-Induced Changes in Cholesterol

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Background—Numerous genetic contributors to cardiovascular disease risk have been identified through genome-wide association studies; however, identifying the molecular mechanism underlying these associations is not straightforward. The Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial of rosuvastatin users identified a sub–genome-wide association of rs6924995, a single-nucleotide polymorphism ≈10 kb downstream of myosin regulatory light chain interacting protein (MYLIP, aka IDOL and inducible degrader of low-density lipoprotein receptor [LDLR]), with LDL cholesterol statin response. Interestingly, although this signal was initially attributed to MYLIP, rs6924995 lies within RP1-13D10.2, an uncharacterized long noncoding RNA.

Methods and Results—Using simvastatin and sham incubated lymphoblastoid cell lines from participants of the Cholesterol and Pharmacogenetics (CAP) simvastatin clinical trial, we found that statin-induced change in RP1-13D10.2 levels differed between cell lines from the tails of the white and black low-density lipoprotein cholesterol response distributions, whereas no difference in MYLIP was observed. RP1-13D10.2 overexpression in Huh7 and HepG2 increased LDLR transcript levels, increased LDL uptake, and decreased media levels of apolipoprotein B. In addition, we found a trend of slight differences in the effects of RP1-13D10.2 overexpression on LDLR transcript levels between hepatoma cells transfected with the rs6924995 A versus G allele and a suggestion of an association between rs6924995 and RP1-10D13.2 expression levels in the CAP lymphoblastoid cell lines. Finally, RP1-13D10.2 expression levels seem to be sterol regulated, consistent with its potential role as a novel lipid regulator.

Conclusions—RP1-13D10.2 is a long noncoding RNA that regulates LDLR and may contribute to low-density lipoprotein cholesterol response to statin treatment. These findings highlight the potential role of noncoding RNAs as determinants of interindividual variation in drug response. (Circ Cardiovasc Genet. 2016;9:223-230. DOI: 10.1161/CIRCGENETICS.115.001274.)

Key Words: cholesterol • genome-wide association studies • long noncoding RNA • low-density lipoprotein cholesterol • simvastatin

Elevated plasma low-density lipoprotein cholesterol (LDLC) is a significant risk factor for cardiovascular disease, the leading cause of death in the world. Statins are the most widely prescribed class of drugs used to lower blood LDLC levels and reduce cardiovascular disease risk. Specifically, statins competitively inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), the rate limiting enzyme in the cholesterol biosynthesis pathway, and thus stimulate hepatic uptake of LDLC through upregulation of the low-density lipoprotein receptor (LDLR).

Although statin efficacy for reducing cardiovascular disease mortality has been well established, there is still substantial residual risk on treatment, and interindividual response with regard to statin effects on cholesterol lowering remains a concern.

Although factors such as smoking status, race, and age have been reported to affect statin efficacy, the pharmacogenetics of statin response is an area of active study. Both clinical trial and population-based cohorts have identified variants in genes such as LPA, APOE, SORT1, HMGCR, and LDLR that were associated with statin effects on LDLC lowering. To date, the largest genome-wide association study performed in a single statin clinical trial was reported in ~7000 participants of the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) clinical trial. Among the gene variants identified from this analysis, Chasman et al reported a sub–genome-wide significant association (P<1×10^-6) between rs6924995 and statin-induced change in plasma LDLC. This particular association was notable because it was attributed to myosin regulatory light chain interacting protein (MYLIP, aka IDOL and inducible degrader of LDLR), an E3 ubiquitin ligase that regulates LDLR stability.

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and thus activity, in response to changes in intracellular cholesterol levels.11 Although rs6924995 is located =10 kb downstream of MYLIP, to date, there is no evidence that rs6924995 affects MYLIP expression levels or function.

Interestingly, rs6924995 is located within RP1-13D10.2, a processed pseudogene. Although RP1-13D10.2 has no known function, many pseudogenes have potential biological functions as noncoding RNAs.12 In addition, long noncoding RNAs (lncRNAs) have been implicated in cardiovascular disease. For example, the relationship between genetic variation at chromosome 9p21 associated with atherosclerotic risk has been attributed to expression changes in an antisense noncoding RNA.13,14 Recently, the lncRNA RP5-833A20.1 was shown to modulate cholesterol homeostasis;15 however, the potential involvement of lncRNAs in statin response has not yet been assessed. Thus, here we sought to determine if RP1-13D10.2 acts as a novel lncRNA regulating cellular cholesterol metabolism, specifically hypothesizing that RP1-13D10.2 may mediate the association between rs6924995 and statin-induced change in LDLC.

Methods

Cell Culture

Lymphoblastoid cell lines (LCLs) from donors of the cholesterol and pharmacogenetics (CAP) population (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained with the Captivating the United Kingdom (UK) Pharmacogenetics (CAP) population (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previously imputed4 were obtained from donors of the CAP (ClinicalTrials.gov ID: NCT00451828)3 with rs6924995 genotypes previous听说过

Table. Clinical Characteristics of Study Participants Split by Race and LDLC Statin Response

|          | White |         | Black |         |
|----------|-------|---------|-------|---------|
|          | High  | Low     | High  | Low     |
| n        | 23    | 21      | 13    | 18      |
| Men      | 48%   | 62%     | 85%   | 44%     |
| BMI      | 27.9±6.3 | 27.5±4.5 | 32.1±6.6 | 30.2±5.6 |
| Age, y   | 50.6±12.1 | 52.7±9.7 | 50.8±10.5 | 55.1±15.0 |
| Before treatment LDLC level, mg/dL | 136±34 | 132±28 | 141±31 | 122±42 |
| LDLC percent change after statin treatment, % | −58.7±4.1 | −22.0±7.6 | −53.9±5.7 | −26.7±7.3 |
| LDLC level change after statin treatment, mg/dL | −80.3±22.4 | −29.3±12.4 | −76.0±18.3 | −33.6±16.4 |

Data are presented as numbers, percentages, or means±SDs. None of these participants were smokers. BMI indicates body mass index; and LDLC, low-density lipoprotein cholesterol.
according to the manufacturer’s protocol. Twenty-four hours before transfection, HepG2 cells were seeded into 6-well plates at a concentration of 7.5 x 10^5 cells/well, and Huh7 cells were seeded into 6-well plates at a concentration of 5.0 x 10^5 cells/well. Cells were transfected for a total of 48 hours with either the pCMV6-EV or either allele of the RP1-13D10.2 overexpression plasmids, and cellular phenotypes were measured after 48 hours.

To measure LDL uptake, cells transfected with the EV-negative control or either allele of the RP1-13D10.2 expression constructs (rs6924995 A or G) were incubated with 10 µg/mL of DiI-LDL (Biomedical Technologies Inc, BT-904) in Eagle’s minimum essential medium media supplemented with 10% fetal bovine serum for 2 hours. Cells were washed twice in PBS and scraped from wells. Levels of DiI-LDL uptake were quantified by fluorescence-activated cell sorting on a BD FACS Calibur Flow Cytometer, and values were calculated using the average of 10,000 gated events. Experiments were performed 4x. Effects of RP1-13D10.2 overexpression on media levels of apolipoprotein B were quantified by ELISA as previously described. To measure the effect of RP1-13D10.2 overexpression on LDLR transcript stability, Huh7 cells were transfected for 48 hours with either the pCMV6-EV or the RP1-13D10.2 overexpression plasmids, treated with 1 µg/mL actinomycin D and harvested >6 hours. Transcript half-life was calculated as described previously.

SREBF1 and SREBF2 knockdown was achieved by 48-hour transfection of 1.5 x 10^5 Huh7 cells/well in 6-well plates using siPORT NeoFX transfection agent (Ambion) with either Silencer Select siRNA (Ambion) or nontargeting control, according to the manufacturer’s protocol. HepG2 cells were incubated with 1 µmol/L GW3965 (Sigma-Aldrich) dissolved in dimethyl sulfoxide for 24 hours before collection for RNA isolation.

Cycloheximide Treatment
Huh7 cells were transfected with RP1-13D10.2 G or A plasmid using GenJet (SignaGen Laboratories) according to manufacturer’s protocol. Cells were incubated with cycloheximide (20 µg/mL) for 24 hours. To quantify total cellular protein, cells were dissolved in CellLytic M (Sigma-Aldrich) and centrifuged at 16,000g. Supernatant was quantitated using the Bio-Rad Protein Assay kit (Bio Rad) and measured on a Synergy H1 microplate reader (Biotek).

Statistical Analyses
Differences between EV and RP1-13D10.2 overexpression (either A or G plasmid) on cellular phenotypes including gene expression, LDL uptake, and media apolipoprotein B were identified using 2-tailed t-tests in which samples within an experimental batch were paired. One-way ANOVA with post hoc 2-tailed paired t-tests was used to identify differences in gene expression levels after cellular incubation under various conditions. Unless otherwise stated, all statistical analyses were performed in GraphPad Prism 6.0.

Results
Statin-Induced Change in RP1-13D10.2 Expression Differs Between High Versus Low LDLC Statin Responders
We first sought to determine if either RP1-13D10.2 or MYLIP gene expression was related to LDLC statin response using simvastatin and sham exposed LCLs from participants of the CAP clinical trial with either high or low LDLC response (Table). Statin-induced change in RP1-13D10.2 expression levels significantly differed between high and low responders in cell lines from both white (23 high responders versus 21 low responders) and black (13 high responders versus 18 low responders) donors. The statin-induced change in RP1-13D10.2 expression in the high responders of both populations was greater than the low responders.

Figure 1. A, Statin-induced change in RP1-13D10.2 expression levels differs between high and low low-density lipoprotein cholesterol (LDLC) responders to statin treatment. CAP lymphoblastoid cell lines from the tails of the white and black LDLC distribution were incubated with 2 µmol/L simvastatin or sham buffer for 24 hours, after which RP1-13D10.2 and myosin regulatory light chain interacting protein (MYLIP) expression levels were quantified by RNA-seq. Because variance stabilization is approximately like a log2 transformation, the approximate fold change was estimated as 2(variance stabilized sham-variance stabilized statin). Although fold changes (mean±SE) are displayed for ease of interpretation, P values were calculated from t tests on the variance stabilized deltas. Transcripts on the + strand are indicated in blue and transcripts on the – strand are indicated in green. B, RP1-13D10.2 is located on chromosome 6 to 10 kb downstream of MYLIP and is proximal to 2 additional processed, uncharacterized pseudogenes, RP1-13D10.3 and RP1-13D10.4.
(1.11±0.06-fold versus 0.90±0.04-fold, respectively, \(P=0.006\) in whites, and 1.21±0.06-fold versus 0.96±0.04-fold, respectively, \(P=0.002\) in blacks). In contrast, we observed no relationship between statin response and change in \(MYLIP\) transcript levels (Figure 1A).

**RP1-13D10.2 Transcript Structure**

RP1-13D10.2 is annotated to the + strand of chromosome 6. As shown in Figure 1B, it is located ≈10 kb downstream of \(MYLIP\), and it is adjacent to 2 processed pseudogenes, RP1-13D10.3 (annotated on the − strand) and MRPL42P2 (aka RP1-13D10.4, annotated on the + strand). On closer inspection of our RNA-seq data, we found evidence of a splice junction joining the annotated 3′ end of RP1-13D10.2 to another exon that partially overlaps RP1-13D10.3 in the antisense direction (Figure II in the Data Supplement). There was no evidence of splicing of either pseudogene to RP1-13D10.4.

We quantified RP1-13D10.2, RP1-13D10.3, and RP1-13D10.4 expression levels in LCLs (n=60) derived from participants of the CAP statin clinical trial after in vitro exposure to 2 µmol/L simvastatin or sham buffer. Because these pseudogenes have no introns, we compared expression levels in cDNA versus no reverse transcriptase controls, prepared for each sample, to account for amplification from residual genomic DNA. RP1-13D10.2 expression was detected in both the statin and the sham-treated cells. In contrast, no expression of the annotated RP1-13D10.3 transcript or RP1-13D10.4 was detected in the statin or sham-treated cells (Figure III in the Data Supplement).

**RP1-13D10.2 Increases LDLR Expression and Stimulates LDL Uptake**

To determine if RP1-13D10.2 affects genes involved in cholesterol metabolism, we transiently transfected Huh7 cells with a plasmid overexpressing the RP1-13D10.2 gene containing either the rs6924995 A or G allele, or pCMV6-entry, a control plasmid that contained the empty vector backbone. Although we identified a novel splice variant of RP1-13D10.2, we chose to focus our functional investigation on the canonical RP1-13D10.2 transcript. After 48 hours, overexpression of RP1-13D10.2 was confirmed by quantitative PCR (Figure IVA in the Data Supplement), and genes involved in cholesterol biosynthesis (\(HMGCR\) and \(HMGCS1\)) and cholesterol uptake (\(LDLR\), \(MYLIP\), and \(PCSK9\)) were quantified (Figure 2A). Overexpression of the RP1-13D10.2 containing the rs6924995 A allele of increased \(LDLR\) transcripts by 2.35±0.51-fold, \(P=0.002\), whereas overexpression of the G allele increased \(LDLR\) transcripts by 2.04±0.57-fold, \(P=0.03\). Notably, these effects seemed to be specific for \(LDLR\) because there were no consistent expression differences in any other genes, including \(MYLIP\).

Similar effects of RP1-13D10.2 overexpression were also observed in a second human hepatoma cell line, HepG2 (Figure IV in the Data Supplement). To verify the functional impact of RP1-13D10.2 overexpression, we tested the effect of overexpression on uptake of DiI-labeled LDL. Consistent with increased expression levels of \(LDLR\), we found that RP1-13D10.2 overexpression increased DiI-LDL uptake with either the ‘A’ allele (1.85±0.08 fold, \(P<0.0001\)) or ‘G’ allele (1.66±0.13 fold, \(P=0.0002\); Figure 2B), as well as

![Figure 2](image-url)
reduced media levels of apolipoprotein B (Figure 2C). Finally, as \textit{LDLR} transcript levels are known to be regulated at the level of transcription\textsuperscript{23} as well as mRNA stability, we tested if \textit{RP1-13D10.2} altered \textit{LDLR} stability using incubation with actinomycin D, but found no evidence of an effect (Figure 2D).

**\textbf{RP1-13D10.2 Affects LDLR Transcript in the Absence of Protein Synthesis}\**

\textit{RP1-13D10.2} is currently annotated as a processed pseudogene by Gencode. However, recent findings have reported that some noncoding RNAs are translated and function as micropeptides, rather than as noncoding RNAs.\textsuperscript{24} \textit{RP1-13D10.2} does have an open reading frame that, if translated, would encode a protein of 97 amino acids with the rs6924995 G allele (Figure V in the Data Supplement). Notably, this putative open reading frame would be disrupted by the rs6924995 A allele, TGG (tryptophan) to TAG (stop), resulting in a protein of 82 amino acids. Thus, to test the possibility that \textit{RP1-13D10.2} functions as a protein, we tested if overexpression in the presence of cycloheximide, an inhibitor of protein synthesis, was able to stimulate increased expression levels of \textit{LDLR}. Although cycloheximide treatment reduced total cellular protein by \approx 50\%, it had no effect on \textit{RP1-13D10.2} induction of \textit{LDLR} expression levels (Figure 3).

To further evaluate the possibility that \textit{RP1-13D10.2} is translated, we used 3 in silico prediction programs, the coding–noncoding index,\textsuperscript{25} the coding potential assessment tool,\textsuperscript{26} and the coding potential calculator.\textsuperscript{27} Both the coding–noncoding index and the coding potential assessment tool analyses indicated that the \textit{RP1-13D10.2} transcript with either the A or G rs6924995 alleles was noncoding, whereas the coding potential calculator analysis indicated there was weak evidence that both A and G allele containing transcripts were coding.

**\textbf{Effects of rs6924995 on RP1-13D10.2 Regulation of LDLR}\**

We observed a consistent trend of slightly larger effect sizes of the A allele versus G allele overexpression on both \textit{LDLR} expression and LDL uptake; however, this difference was not statistically significant (\textit{P}=0.10 for LDL uptake). Because this trend was observed in 3 different hepatoma cell lines (Figure IVC in the Data Supplement), we hypothesized that our model of extreme \textit{RP1-13D10.2} overexpression (\approx 10\textsuperscript{10}-fold increase) may obscure the potential differences between the 2 alleles. With reduced levels of \textit{RP1-13D10.2} overexpression (\approx 10\textsuperscript{7}-fold) we continued to observe a similar trend of smaller increases in \textit{LDLR} upregulation with the G allele versus A allele; however, this difference did not achieve statistical significance (\textit{P}=0.06, Figure IVD and IVE in the Data Supplement). Further reductions in the degree of \textit{RP1-13D10.2} overexpression failed to produce consistent stimulation of \textit{LDLR} transcript with either allele.

**\textbf{Figure 3. \textit{RP1-13D10.2} stimulation of LDLR does not require protein synthesis.}\**

Huh7 cells (n=3) were transfected with a construct containing either \textit{RP1-13D10.2} rs6924995 G alleles or an empty vector (EV) control in duplicate, after which 1 aliquot was treated with 20 \textmu g/mL cycloheximide for 24 hours, and \textit{LDLR} transcript was quantified by quantitative polymerase chain reaction. Values shown are means+SE.

**\textbf{Figure 4. \textit{RP1-13D10.2} expression levels are sterol regulated.}\**

\textbf{A}, \textit{RP1-13D10.2} transcript levels were quantified in HepG2 cells were exposed to 4 culture conditions: fetal bovine serum (FBS, control), 48-hour incubation with 2-\textmu mol/L simvastatin+10\% lipoprotein–deficient serum (LPDS), 24-hour incubation with statin+LPDS after which low-density lipoprotein cholesterol (LDLC) or 25HC was added back and incubated for an additional 24 hours. The experiment was repeated 6x, with means+SE shown. Statistically significant differences in gene expression were assessed using 1-way ANOVA (\textit{P}=0.01), with 2-tailed paired t tests used to identify differences between groups. \textbf{B}, \textit{RP1-13D10.2} transcript levels were quantified after \textit{SREBF1} and \textit{SREBF2} knockdown in Huh7 cells. \textbf{C}, \textit{RP1-13D10.2} and myosin regulatory light chain interacting protein (\textit{MYLIP}) transcript levels were quantified in HepG2 cells after 24-hour incubation with 1 \textmu mol/L GW3965 (n=6).
RP1-13D10.2 Expression and rs6924995 Genotype

Given the fact that rs6924995 is contained within RP1-13D10.2, we next sought to determine if this single-nucleotide polymorphism was associated with expression levels of the pseudogene. We observed a suggestive association between rs6924995 genotype and RP1-13D10.2 expression in both the statin and the sham-treated cells, with trends toward greater RP1-13D10.2 expression observed in the GG homozygotes (Figure VI in the Data Supplement). In contrast, there was no relationship between rs6924995 and MYLIP expression levels (Figure VI in the Data Supplement). Closer examination of genomic region between MYLIP and RP1-13D10.2 found that rs6924995 is within a small block of linkage disequilibrium that contains RP1-13D10.2, but not MYLIP (Figure VII in the Data Supplement).

RP1-13D10.2 Expression Levels Are Sterol Regulated

Many genes that affect cholesterol metabolism are themselves subject to sterol regulation. To test if RP1-13D10.2 transcript levels were affected by changes in intracellular sterol content, we exposed HepG2 cells to conditions of extreme sterol depletion (2 μmol/L simvastatin + 10% lipoprotein-deficient serum) for 24 hours, after which we added-back LDLC. Sterol depletion reduced RP1-13D10.2 expression levels 0.43±0.12-fold (Figure 4A). LDLC add-back not only reversed this effect but also highly induced RP1-13D10.2 expression.

Sterol response element–binding proteins and LXR are well-known transcription factors that mediate cellular response to changes in intracellular sterols. RP1-13D10.2 expression levels were significantly reduced after SREBF2 knockdown in HuH7 cells (0.5±0.11-fold, P=0.03, Figure 4B; Figure VIII in the Data Supplement), no effect was observed with SREBF1 knockdown. We also found evidence that RP1-13D10.2 is regulated by LXR as incubation of HepG2 with GW3965, an LXR agonist, increased RP1-13D10.2 expression levels (1.4±0.13-fold, P=0.02, Figure 4C). Notably, these effects were much more modest compared with MYLIP, a known LXR target gene, which was increased 5.8±0.46-fold (P<0.0001).

Discussion

We have examined RP1-13D10.2, an lncRNA containing a single-nucleotide polymorphism, rs6924995, reported to have a sub–genome-wide association with LDLC response to rosuvastatin. Here, we report that RP1-13D10.2 overexpression increases transcript levels and activity of LDLR, which encodes the major receptor for uptake of plasma LDLC. RP1-13D10.2 expression seems to be sterol regulated, and notably we observed a relationship between interindividual variation in the magnitude of this regulation with statin-induced changes in LDLC from a panel of LCLs derived from participants of a statin clinical trial. In particular, statin incubation increased RP1-13D10.2 expression levels in cell lines from both whites and blacks with high LDLC response to statin treatment, whereas either no change or reduced RP1-13D10.2 expression was detected in cell lines from donors with low LDLC response. Together, these findings support the identification of RP1-13D10.2 as a novel marker, and possibly determinant, of variation in statin efficacy for plasma LDLC lowering. Although lncRNAs have been well established to play a role in cardiovascular biology and disease, to our knowledge, RP1-13D10.2 is the first lncRNA that has been identified to play a role in statin response.

rs6924995 was first reported to be associated with LDLC response to statin in the JUPITER placebo-controlled trial of rosuvastatin (20 mg/d) response. The A allele was associated with both greater absolute (β=4.1, P=5.3E-07) and fractional (β=3.8, P=1.4E-06) LDLC reduction in individuals with genetically confirmed European ancestry. The Heart Protection Study, a five-year trial of 3895 self-reported Caucasians prescribed 40 mg simvastatin/d, failed to replicate this association with LDLC statin response.29 In addition, the largest genome-wide meta-analysis of LDLC response to statin treatment published to date, comprising 18,596 subjects from clinical trial and population-based cohorts, did not identify this single-nucleotide polymorphism; however, the report did not include a direct test for replication of this locus. This lack of replication may be because of the unique nature of the JUPITER study population. Statins are traditionally prescribed to individuals with hypercholesterolemia; in contrast JUPITER was composed of participants with relatively normal levels of LDLC (<130 mg/dL), but who had high measures of inflammation (C-reactive protein ≥2 mg/dL).

The association between rs6924995 and LDLC response to statin treatment was originally attributed to MYLIP (aka IDOL and inducible degrader of LDLR), a known regulator of LDLR protein levels. Here, we failed to identify a relationship between rs6924995 genotype and MYLIP transcript levels in LCLs. In contrast, we found suggestions of an association between RP1-13D10.2, an lncRNA that contains rs6924995 and is located 10 kb downstream from MYLIP, expression levels with rs6924995 genotype. In addition, although we were unable to verify a genotype difference in RP1-13D10.2 effects on LDLR, the trend of slightly greater stimulation of LDLR transcript with the RP1 A allele constructs versus the G allele constructs is consistent with the genome-wide association studies association between the rs6924995 A allele and greater LDLC lowering on statin treatment. Thus, additional study is necessary to ascertain the true relationship between rs6924995 and RP1-13D10.2 transcript levels, transcript structure, and activity.

We observed no direct effects of RP1-13D10.2 overexpression on MYLIP transcript levels. Although we did not test for an effect of RP1-13D10.2 on MYLIP protein levels, MYLIP is an E3-dependent ubiquitin ligase complex that mediates sterol-dependent degradation of LDLR protein, thus it is unlikely that the effects of RP1-13D10.2 on LDLR transcript levels are mediated by MYLIP. Although further study will be required to absolutely discount a relationship of either rs6924995 or RP1-13D10.2 to MYLIP, these findings suggest the intriguing possibility that RP1-13D10.2 and MYLIP may be mechanistically independent regulators of LDLR activity that happen to be in close proximity to one another, similar to other known clusters of lipid-related genes (ie, APOC3 and APOA5, SREBF2 and mir-33a).

We found that RP1-13D10.2 expression levels were increased with statin treatment in LCLs from donors with high LDLC response to statin treatment. Sterol response element–binding protein 2 (gene name SREBF2) is a well-known
transcription factor that is activated by conditions of sterol depletion, such as in vitro statin exposure. In human hepatoma cell lines, we found that SREBF2 knockdown reduced RP1-13D10.2 expression levels, consistent with the likelihood that RP1-13D10.2 may be an SREBF2 target gene. In addition, RP1-13D10.2 expression levels were also increased after incubation with an LXR agonist, suggesting that it may also be an LXR target gene. Notably, MYLIP is a well-known LXR target gene, and thus the close proximity between RP1-13D10.2 and MYLIP may allow for shared transcription factor regulatory sequences. Paradoxically, these 2 genes would be expected to oppose one another, as LXR-induced expression of RP1-13D10.2 would increase LDLR activity, whereas LXR-induced expression of MYLIP would stimulate LDLR decay. However, this is similar to the well-known phenomena in which SREBF2 both induces LDLR transcription, whereas stimulating expression of a factor, PCSK9, that promotes LDLR protein decay.31

A question that remains is the precise mechanism by which RP1-13D10.2 specifically increases transcript levels of LDLR. Our observation that RP1-13D10.2 upregulates LDLR after cycloheximide incubation supports the likelihood that RP1-13D10.2 functions as an lncRNA; however, as the cells were treated with cycloheximide 24 hours after transfection with the RP1 plasmid, these findings cannot exclude the possibility that RP1-13D10.2 acts as a highly stable protein that persists after inhibition of protein synthesis. However, when combined with our in silico analysis that does not support the coding potential of RP1-13D10.2, our findings strongly support the likelihood that RP1-13D10.2 functions as a noncoding RNA.

There are 4 major described functions of lncRNAs (recently reviewed by Uchida and Dimmel):28 (1) imprinting—the lncRNA directly inhibits expression of a proximal locus; (2) guide molecules—the lncRNA recruits functional proteins, often epigenetic or transcription factors, in a transacting manner; (3) enhancer activation—the lncRNAs is transcribed from the site of an enhancer element and aids in enhancer activity; (4) molecular sponges—the lncRNA binds miRNAs, disrupting miRNA inhibition of mRNAs. Endogenous levels of RP1-13D10.2 are low compared with LDLR, thus it is unlikely that RP1-13D10.2 functions as a molecular sponge, which often requires similar stoichiometry of the effector and target molecules.29 In addition, we found that RP1-13D10.2 overexpression increases LDLR transcript levels without affecting LDLR transcript stability, suggesting that RP1-13D10.2 enhances LDLR transcription. However, RP1-13D10.2 is located on chromosome 6, whereas LDLR is located on chromosome 19, thus RP1-13D10.2 does not likely affect LDLR through either imprinting or changes in LDLR enhancer activity. Thus, the most probable function of RP1-13D10.2 is as a guide molecule. Notably, neither moderate nor high expression is required for this function as the biological effects of even a lowly expressed lncRNA may be amplified through a signaling cascade.28

One of the major findings of the large transcriptomic projects of the past decade is the widespread transcription of the human genome, and recent estimates using RNA-seq data suggest that ≈80% of the genome is transcribed, with many of these transcribed sequences representing ncRNAs.22,23 Using a combination of gene expression and functional studies, here we identify the lncRNA RP1-13D10.2 as a novel marker, and possible determinant, of LDLC response to statin treatment that regulates LDLR. Thus, our findings illustrate the potential of noncoding regulatory RNA as a determinant of variability in drug response. In addition, as genome-wide association studies identified single-nucleotide polymorphisms are most often annotated based on the their proximity to protein coding genes, these results demonstrate the importance of functional validation studies not only as an alternative approach for validating pharmacogenetic associations, but also for ensuring the correct annotation of genome-wide association study findings.

Acknowledgments
We thank Jerome I. Rotter and Yi-D. Chen for creation of the cholesterol and pharmacogenetics (CAP) lymphoblastoid cell lines; and Ronald M. Krauss for his insightful discussions and critical review of this article. This study would not have been possible without the contributions of the CAP participants.

Sources of Funding
This work was supported by the National Institutes of Health (NIH): U19 HL069757 (Dr Medina, Dr Mitchel, Dr Dosé, and D. Naidoo), R01 HL104133 (Drs Medina and Dosé), P50 GM115318 (Dr Medina) and NIH Pharmacogenomics Research Network RNA Sequencing Project (GM161390), as well as the American Heart Association 12POST10430005 (Dr Theusch).

Disclosures
None.

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**CLINICAL PERSPECTIVE**

Statins are among the most prescribed drugs in the United States, used to decrease low-density lipoprotein cholesterol (LDL-C) levels for the prevention and treatment of cardiovascular disease. However, response to statins is variable, and many patients are left with insufficient LDL-C-lowering, despite treatment. Genome-wide association studies have identified a number of DNA variants that are associated with this interindividual variation. Although simply identifying variants may be sufficient for the development of diagnostics, understanding the molecular mechanisms underlying these associations is essential for fully leveraging these findings into new biology that may inform advances in biomedical research. Previously, a sub–genome-wide association was observed between rs6924995 and rosuvastatin response in the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) clinical trial. Located downstream of myosin regulatory light chain interacting protein, a gene known to regulate the major LDL receptor, rs6924995 was originally annotated as the myosin regulatory light chain interacting protein locus. However, rs6924995 is located within the uncharacterized long noncoding RNA, *RP1-13D10.2*, thus here we explored the contribution of this long noncoding RNA to LDL-C statin response. Using cell lines established from participants of a statin clinical trial with either high or low LDL-C response, we found significant differences in statin-induced expression in *RP1-13D10.2* expression between the two groups, while no change was observed with myosin regulatory light chain interacting protein expression levels. Furthermore, *RP1-13D10.2* overexpression in hepatoma cell lines up-regulated the major LDL receptor and increased uptake of LDL. Our data support the hypothesis that *RP1-13D10.2* is a novel marker, and possible determinant, of LDL-C response to statin treatment, and highlight the importance of functional studies for annotation of genome-wide association studies identified loci.