Genome-wide screen for modulation of hepatic Apolipoprotein A-I (ApoA-I) secretion

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Background: Increasing HDL-c through ApoA-1 expression is hypothesized to reduce cardiovascular deaths significantly.

Results: Genes that regulate hepatocyte ApoA-I secretion were identified using 21,789 siRNAs.

Conclusion: Forty genes of interest were confirmed as regulators of ApoA-1 production by hepatocytes.

Significance: This study provides functional genomics-based data for exploring new mechanisms by which ApoA-I levels may be regulated.

SUMMARY

Control of plasma cholesterol levels is a major therapeutic strategy for management of coronary artery disease (CAD). Although reducing LDL cholesterol (LDL-c) levels decreases morbidity and mortality, this therapeutic intervention only translates into a 25-40\% reduction in cardiovascular events. Epidemiological studies have shown that high LDL-c level is not the only risk factor for CAD; low HDL cholesterol (HDL-c) is an independent risk factor for CAD. Apolipoprotein AI (ApoA-I) is the major protein component of HDL-c that mediates reverse cholesterol transport from tissues to the liver for excretion. Therefore, increasing ApoA-I levels is an attractive strategy for HDL-c elevation. Using genome-wide siRNA screening, targets that regulate hepatocyte ApoA-I secretion were identified through transfection of 21,789 siRNAs into hepatocytes whereby cell supernatants were assayed for ApoA-I.

Approximately 800 genes were identified and triaged using a convergence of information, including genetic associations with HDL-c levels, tissue specific gene expression, druggability assessments, and pathway analysis. Fifty-nine genes were selected for reconfirmation; forty genes confirmed. Here we describe the siRNA screening strategy, assay implementation and validation, data triaging, and example genes of interest. Among the genes of interest are known and novel genes encoding secreted enzymes, proteases, GPCRs, metabolic enzymes, ion transporters and proteins of unknown function. Repression of farnesyltransferase (FNTA) by siRNA and the enzyme inhibitor, Manumycin A, caused elevation of ApoA-I secretion from hepatocytes and from transgenic mice expressing hApoA-I and cholesterol ester transfer protein transgenes. In total, this work underscores the power of functional genetic assessment to identify new therapeutic targets.

Coronary artery disease (CAD) is one of the major causes of morbidity and mortality worldwide. About 800,000 new cases of myocardial infarction (MI) and 500,000 recurrent MIs occur each year in the US; whereby, dyslipidemia is a major risk factor for CAD and MI. Although lowering LDL-c is a primary therapeutic approach through administration of statin drugs, effective treatment of CAD and
dyslipidemia should include treatment of low HDL-c levels. Low HDL-c is associated with a substantial increase in risk of CAD (1), and several epidemiological and secondary prevention trials strongly support the hypothesis that agents that increase HDL-c and reduce triglycerides produce significant reduction in death due to cardiovascular events, including nonfatal and fatal myocardial infarction (1). The VA-HIT trial (2) demonstrated that gemfibrozil, a PPARα agonist, elevated HDL-c and lowered triglycerides without lowering LDL-c and provided a 22% reduction in relative risk of major coronary events in patients with coronary artery disease. Fenofibrate, another PPARα agonist, has been shown in the Diabetes Atherosclerosis Intervention Study to be effective at lowering triglycerides and LDL cholesterol and increasing HDL cholesterol in patients with non-insulin dependent diabetes mellitus (NIDDM). Furthermore, treatment with fenofibrate was associated with a reduction in angiographic progression of coronary artery disease in NIDDM (3). Indeed, the National Cholesterol Education Program recommends that HDL-c be screened as an independent risk factor for CAD. At present, an optimum therapy for the treatment of low HDL-c is unavailable. The marketed weak PPARα agonists (fibrates) produce a moderate (10-15%) elevation in plasma HDL-c, and the newer class of promising Cholesterol Ester Transfer Protein (CETP) drugs are still in clinical development.

HDL-c is a small, high density circulating lipoprotein particle that is comprised of phospholipid, cholesterol, and triglycerides with myriad associated proteins. The major structural apolipoprotein associated with HDL-c is apolipoprotein A-I (ApoA-I). ApoA-I is synthesized and secreted by the liver and intestine, and upon secretion, lipid-poor ApoA-I and nascent HDL particles must acquire lipid to form mature HDL-c particles. Mechanistically, ApoA-I mediates the acquisition of phospholipids and unesterified cholesterol through cellular efflux of phospholipids and cholesterol via the transporter adenosine triphosphate-binding cassette protein A-1 (ABCA-1). Subsequently, Lecithin-Cholesterol Acetyltransferase (LCAT) is activated by ApoA-I to transfer fatty acid from phosphatidylcholine to unesterified cholesterol producing cholesteryl ester, which drives the movement of unesterified cholesterol into HDL. This reverse cholesterol transport mechanism is believed to protect against or resolve atherosclerosis by relieving the cholesterol burden from macrophages in the vasculature and returning the cholesterol to the liver for catabolism through the bile acid synthetic route. The importance of ApoA-I in protecting vessels against cholesterol-laden atherosclerotic plaques was underscored by significant reduction in atheroma volume of patients suffering from acute coronary syndrome upon infusion of an ApoA-I variant peptide mimetic for only five weeks (4). Thus, discovering molecular mechanisms involved either in increased expression, stability, or secretion of ApoA-I from liver would provide potential therapeutic targets for elevating levels of circulating HDL-c.

Our approach to identify new mechanisms that are involved in ApoA-I production and secretion was to query the whole genome through siRNA knockdown technology coupled with measuring hepatocyte production of ApoA-I. Genes of interest found by elevation of ApoA-I in culture media upon mRNA knock-down were prioritized using a convergence of information, including literature-based human genetic associations with HDL-c levels, liver- and intestine-specific expression patterns, druggability assessments, and pathway analysis. Known and novel genes encoding secreted enzymes, proteases, GPCRs, metabolic enzymes, ion transporters and proteins of unknown function were identified and evaluated as potential new therapeutic targets. One gene of interest, farnesyltransferase (FNTA), was further tested for its role in controlling hepatic ApoA-I secretion by using siRNA and a compound inhibitor, Manumycin A in cell-based and preclinical in vivo model of human lipoprotein metabolism.

EXPERIMENTAL PROCEDURES

Cell culture: The hepatocarcinoma cell line HepG2 (ATCC, Manassas, VA) was cultured in 3:1 DMEM:F12 (Invitrogen, Carlsbad, CA), 10% FBS (Invitrogen, Carlsbad, CA), and 1X pen-strep (Invitrogen, Carlsbad, CA). Confluent cells were treated with manumycin A (Sigma, St. Louis, MO) at a variety of concentrations.

Transfection of siRNA library: The screening conditions were reverse transfection of 5000 cells/well with (0.25 μl/well) Dharmafect 3 and 50 nM Dharmacon Smartpool siRNAs (4
individual siRNAs, 12.5 nM each; (Dharmacon, Lafayette, CO). Reverse transfection occurred on day 1, followed by a 72 hour incubation with siRNA. To determine the impact of siRNA knockdown on secretion of ApoA-I, media were changed at 72 h post transfection and conditioned medium was analyzed 24 h later for ApoA-I by ELISA (MABtech, Mariemont, OH) and viability by Cell-Titer Glo (CTG, Promega, Madison, WI) according to manufacturer’s protocols. Culture media were diluted 1/20 for ApoA-I ELISA measurements.

ApoA-I assay validation: A 3-day, 3-plates per day statistical validation study was performed according to the Eli Lilly and Company assay optimization and validation procedures to assess ApoA-I signal separation between a non-targeting control (NTC) and both a positive (ADRA1A) and a negative control (ApoA-I) (5). Two Z-primes were calculated from the controls on each plate with an acceptance criterion of \(Z^* \geq 0.4\). Acceptance criteria were also applied to individual plates during the screen as follows: ADRA1A to NTC ratio \(\geq 1.5\), ADRA1A coefficient of variation (CV) \(\leq 25\%\), NTC CV \(\leq 25\%\), and \(Z^*\) for NTC and ApoA-I separation \(\geq 0.3\).

Primary Screen – In partnership with Dharmacon Screening Services (Lafayette, CO), the conditions described above were validated in full screen scale. Subsets of the whole genome were screened sequentially starting with the GPCR (516) and Kinase (800) siRNA libraries, followed by the Druggable Genome library (7,317) and screening completed with the Rest of Genome (ROG) library (14,000). In total, 21,789 human genes were targeted with Dharmacon Smartpools (4 siRNA/well) in triplicate where 80 Smartpools were evaluated per plate, and 880 plates were used to screen the genome libraries in triplicate.

Statistical Analysis - Genes were delivered to triplicate plates in order by family, and as such, genes on a particular plate could be highly correlated. Therefore, the typical approach of estimating within-plate variability and identifying hits relative to the variability on each plate was not used. In order to avoid potential plate bias and make best use of the replicates, ApoA-I levels were instead analyzed by a model that averages across replicate plates to obtain an estimate of gene-to-gene variability for each run. The model was applied to within-plate fold-changes relative to NTC to remove plate effects. The run-specific estimates of gene-to-gene variability were used to calculate Z-scores that express a particular gene’s fold-change relative to NTC in units of standard deviation. Genes with Z-score > 2 or < -2 were considered hits. Cell viability was also expressed as fold-change relative to NTC, and hits with fold-change < 0.6 or > 1.4 were considered to have significantly altered cell viability so were excluded.

FNTA Targeting and Inhibition: HepG2 cells were transfected with 30 nM of siRNA. Dharmacon siRNA1 D-008807-02 5’GAAAGUGCAUGGAACUAUU; siRNA2 D-008807-03 5’GAAAUGACUCACCAACAA; siRNA3 D-008807-04 5’CCAAAGAUACUGCUGUAAU; siRNA4 D-008807-18 5’UGCACAUAGGCGAGUCAG targeting FNTA mRNA (accession NM_001018677). Cells were also transfected with ADRA1 siRNA smartpool M-005419-00 (5’GCAGAAAGCAGUCUUCCCAA, 5’UGAGCGCUCCUGAGGAGUA, 5’UGACAAAGAACCAUCAAGUU, 5’GACCAUCCUCUGUACCA) targeting ADRA1 mRNA (accession NM_000680), ApoA-I siRNA SMARTpool M-010994-00 (5’GUACGUGGAUGUGCUAAA, 5’UGAGCGCUCCUGAGGAGUA, 5’GGGAUAACCUGGAAAAGGA, 5’ UAAAGCUCCUUGACAACUUG targeting ApoA-I mRNA (accession NM_000039) or non-targeting control siRNA pool #2 (D-001206-14-05), using Dharmafect 3 transfection reagent, according to manufacturer’s instructions. Media were changed at 72 h post transfection, and conditioned medium was analyzed 24 h later for ApoA-I by ELISA (MABtech) and viability by Cell-Titer Glo (CTG, Promega).

Overexpression of FNTA protein was conducted by transient transfection of full length human full-length farnesyltransferase cDNA MHS1010-74254 Human MGC Verified FL cDNA (IRAT) clone ID 3850453 (Thermo Fisher, Waltham, MA) for 96h using FuGeneHD at a ratio of 4.5:1 (Promega, Madison, WI) into HepG2 cells.

Quantitative PCR analysis- Total RNA was isolated using the RNeasy Plus kit from (Qiagen, Valencia, CA). One microgram of total RNA was converted to cDNA using a High Capacity cDNA
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Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Quantitative PCR was performed by employing a standard curve method using a 7900HT instrument (Applied Biosystems, Carlsbad, CA). Twenty-microliter PCRs were prepared containing 1x Universal master mix (catalog number 4305719, Applied Biosystems, Carlsbad, CA), and either 1x FNTA gene expression assay (Hs00357739_m1 Applied Biosystems, Carlsbad, CA), ApoA-I gene expression assay (Hs00163641_m1, Applied Biosystems, Carlsbad, CA) or beta-Actin gene expression assay (Hs99999903_m1) and 4 µl of template cDNA diluted 1:100 in 10 mM Tris (pH 8.0). PCR conditions for FNTA and ACTB were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The data from FNTA were normalized to the ACTB and calibrated relative to nontargeting control.

Mass Spectrometry determination of ApoA-I and ApoB levels: Apolipoproteins from the culture media were captured by the affinity resin, Liposorb as previously described with modifications (6,7). Briefly, 400 µL of HepG2 cell supernatants were spiked with 1.67 µg of 15N-labeled human ApoA4 as internal standard, then incubated in the presence of 4 mg of Liposorb for 30 minutes at 4°C with constant shaking. Unbound proteins were removed by centrifugation of the Liposorb suspension through 0.22 µm Captiva filter plate at 2000 RPM for 5 minutes, followed by two washes with 100 mM ammonium bicarbonate containing 5 mM EDTA (ABCE). After resuspension of the Liposorb, proteins were denatured by incubation with 8M urea in ABCE for 15 min at 55°C, then digested with trypsin-gold overnight at 37°C with constant shaking (digestion mixture contained 2 ug of Trypsin-gold, 1.6 M urea and 0.01% NP-40 in ABCE), after which samples were filtered through 0.45 µm membrane filter before analysis by LC-MS/MS.

Tryptic peptides were separated by an HPLC system (Thermo Finnigan, Waltham, MA) on a C18 XBridge column (2.5 µm, 2.1 x 50 mm) using the two-solvent gradient system (solvent A, 0.1% formic acid in H2O; solvent B, 0.1% formic acid in acetonitrile) consisting of the following step gradients maintained at 50°C: 100% A at 200 µL/min for 1 min, 10% B at 200 µL/min for 5.1 min, 15% B at 200 µL/min for 5 min, 30% B at 200 µL/min for 3 min, 35% B at 200 µL/min for 3.3 min, 80% B at 200 µL/min for 0.1 min, 80% B at 600 µL/min for 0.9 min, 100% A at 600 µL/min for 1.5 min, followed by 100% A at 200 µL/min for 0.1 min. Two peptides from ApoA1 (DYSQFEGSALGK, ApoA1-52; AKPALEDLR, ApoA1-231), and two peptides from ApoB (IADFELPTIIVPQIIEIPSK, ApoB-3847; FSVPAGIVPSFQALTAR, ApoB-3869) were simultaneously measured along with one peptide from 15N-ApoA4 (LEPYADQLR, h_N15A4_135), which was used for normalization of differences in sample recovery and instrument performance.

Positive ion mass spectrometry was obtained using an LTQ ion trap mass spectrometer equipped with an ESI source (Thermo Finnigan) in multiple reaction monitoring (MRM) mode. The entire effluent of the column was directed to the ESI source between 2.5 and 17.4 min of HPLC run while the rest was diverted away from the mass spectrometer. The m/z selection values for each peptide are given within parentheses as indicated (m/z value for the parent ion/isolation window → m/z values for each daughter ion/isolation window in mass units): apoA1_52 (701.26/4 → 562.29/3, 809.42/4, 1024.51/4), apoA1_231 (507.09/3 → 814.45/4, 726.38/4, 288.70/3), apoB_3847 (1234.46/4 → 1467.85/4, 1354.76/4, 1779.05/4), apoB_3869 (938.11/4 → 991.54/4, 771.95/4, 1203.69/4), h_N15A4_135 (559.58/3 → 437.69/3, 874.37/4, 245.63/3).

For data analysis the peak areas of ApoA1 and ApoB peptides were calculated using Xcalibur 2.1 peak integration and curve fitting software normalized to the internal standard. Normalized peak area for each peptide was converted as percentage of that of the same peptide in the nontargeted cell control, and the average value of the two peptides for the same protein was reported along with percent difference between the two peptides.

Western blotting: HepG2 cells were transfected as described and protein isolated by lysis in 1% SDS buffer containing fresh 100ul (1X) Halt protease inhibitor (Thermo Fisher, Waltham, MA) and 100ul (1X) Halt phosphatase inhibitor (Thermo Fisher, Waltham, MA). Samples were heated at 95°C for 5 min, then sonicated. Samples were quantified with the BCA protein kit (Thermo Fisher, Waltham, MA) prior to dilution.
and addition of 1X NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA) containing 10% beta mercaptoethanol. Samples were heated at 95 °C for 5 min., centrifuged, and loaded along with SeeBlue Plus2 prestained protein ladder (Invitrogen LC5925). Samples (15 ug) were run on Tris-Glycine-4-20% gels (BioRad 567-1094) in 1x Tris-Glycine/SDS running buffer (BioRad 161-0732). Samples were transferred for 6.5 min with the Invitrogen iblot blotting unit with nitrocellulose. Blots were blocked in 5% milk in PBS/0.1% Tween 20 for 30 min at room temperature. Blots were probed with FNTA (Epitomics Burlingame, CA), GAPDH rabbit monoclonal IgG antibody (Cell Signaling, Danvers, MA) or ADRA1A antibody (Epitomics, Burlingame, CA) diluted at 1:1000, 1:5000, and 1:1000 respectively overnight at 4°C in 5% milk in PBS/0.1% Tween 20. Blots were washed 4 times for 10 min each with PBS/0.1% Tween 20 then incubated with HRP-conjugated goat anti-rabbit secondary antibody diluted 1:5000 (Jackson Immunoresearch Labs, West Grove, PA) for 1 hour at room temperature. Blots were washed 4 times for 10 min each at room temperature in PBS/0.1% tween 20 then developed with Pierce SuperSignal West Pico kit (Thermo Fisher, Waltham, MA) and imaged on a LAS-4000 Luminescent Analyzer (GE Healthcare Lifesciences, Piscataway, NJ).

**Manumycin A treatment of HepG2 cells:** HepG2 cells were seeded into 24 well plates at 200,000 cells per well and were grown for 48 h before treatment. The culture media were removed, and a dose response of manumycin A dissolved in DMSO was added to fresh media and applied to the cells. The cells were incubated for 4 hours, and ApoA-1 levels in the media were determined by ELISA.

**Manumycin A treatment of mice:** All animals received deionized water and 2014 Teklad Global Diet (Harlan, Indianapolis, IN) *ad libitum* and were maintained in accordance with the Institutional Animal Use and Care Committee of Eli Lilly and Company and the NIH Guide for the Use and Care of Laboratory Animals. The treatment with farnesyltransferase inhibitor, manumycin A (5 mg/kg body weight dissolved in 0.4% dimethyl sulfoxide, phosphate buffered saline, subcutaneously 3 times per week; Sigma, St. Louis, MO), or vehicle alone was commenced at 7 weeks of age in hApoA-I x hCETP transgenic mice (Taconic, Germantown, NY). The treatment with manumycin A was continued for 14 days. At day 1 and day 14, blood samples were obtained to measure serum hApoA-I levels by ELISA (MABtech, Mariemont, OH) n=6 mice per group.

**RESULTS**

*Genome-wide siRNA screen:* Several factors were considered in selecting the cell model for the functional genomics screen. Since the main sources of ApoA-I in human are the liver and small intestine, a human cell line from one of these tissue types to identify modulators of ApoA-I secretion was preferred. In addition, ease and reproducibility of culturing and expanding the cell line to create a single lot of cells for high throughput screening (HTS) was considered. Lastly, the cell line needed to be amenable to reverse transfection methods that integrate well with automated HTS methodologies. Given these requirements, the human hepatocarcinoma cell line HepG2 was selected for assay development. HepG2 cells secrete ApoA-I and have been used successfully in other HTS applications (8).

Once an appropriate cell line was selected, optimal growth and siRNA transfection conditions were determined and validated in a 96-well format. As the first proof-of-concept for the assay, a Smartpool siRNA targeting ApoA-I was tested for the ability to repress both ApoA-I mRNA and ApoA-I protein in the culture medium. The ApoA-I Smartpool reduced ApoA-I mRNA levels by 88% and repressed secreted ApoA-I protein by 97% as compared to the neutral, non-targeting control (NTC) siRNA (Fig. 1A). Two siRNAs that modulated ApoA-I secretion in HepG2 cells either positively or negatively over the control were identified. Adrenergic Receptor Alpha 1A (ADRA1A) Smartpool siRNA was found to modulate ApoA-I protein secretion in the positive direction, and therefore served as the condition defining the “maximal” signal in each assay. When transfected into HepG2 cells, the ADRA1A Smartpool siRNA caused a 92% reduction in ADRA1A mRNA and an 86% reduction in ADRA1A protein levels (Fig. 1B). Knockdown of ADRA1 caused a 75% increase in ApoA-1 secretion as compared to the NTC siRNA. ADRA1A is a G-protein coupled receptor that is expressed in liver, signals through the Gq/11...
family of G-proteins, is activated by epinephrine and norepinephrine, and mediates calcium release upon ligand binding. The mechanism by which endogenous levels of ADRA1A expression appears to increase ApoA-I secretion is currently unknown. As shown previously, the Smartpool targeting ApoA-I mRNA itself decreased secretion and served as the definition of “minimal” signal in each assay, and the ADRA1 siRNA Smartpool induced ApoA-I secretion by ≥50% in a three-plate, three-day reproducibility study (Fig. 1C). Each assay plate in the genome-wide screen contained a specific configuration of control wells of NTC, ApoA-I and ADRA1 Smartpools as a measure of technical success. Secreted ApoA-I levels were normalized to the median of the NTC on each plate. Data were reported as fold-change from the NTC.

To ensure that the assay was robust with sufficient dynamic window, the overall requirement for the signals was that the raw signals had to show sufficient separation between the NTC and the ApoA-I and ADRA1 controls, and the fold-change from NTC had to be sufficiently reproducible. Using the conditions described above, significant separation of ApoA-I levels between the NTC siRNA and ApoA-I negative control siRNA was observed with Z-primes (Z’s) well above 0.4. Sufficient separation between the NTC siRNA and ADRA1 positive control siRNA was observed with nearly all Z’s above 0.4. While some plates illustrated “edge effects” and other patterns, none were >20% in magnitude. The application of the individual plate acceptance criteria resulted in rejection of just 10 plates for the entire screen of 880 plates, and in no case was more than one of the triplicate plates rejected.

Two Z-scores that measure how far a particular gene’s response is above or below the non-targeting siRNA control (NTC) relative to the variability in gene response were implemented to describe the change of ApoA-I protein levels. This approach expresses a particular gene’s response relative to the NTC in units of standard deviation so that a Z-score of 2 (-2) means a particular gene’s response is two standard deviations above (below) the NTC. Only Z-scores > 2 or < -2 for the analysis were pursued. Hits that significantly altered cell viability, based on the normalized CTG values, were excluded if the values were less than 0.6 or greater than 1.4 (0.6 ≤ CTG ≤ 1.4).

**Hit Triage:** The distribution of siRNA activity was continuous whereby 756 of the targets met the criteria of z-score ±2 where the highest z-score was 6.8, and the lowest was -2.4 (Fig. 1D). Confirmation of the activity of these Smartpools was conducted by a replicate analysis, and 85% of the original hits were reproducibly active. Genes of interest were further refined by eliminating low-probability candidate genes based on biological criteria, such as tissue expression patterns. Informatic resources such as Proteome, GNF Human Atlas, and Oncogenomics enabled filtering of genes that are expressed in liver and/or intestine. The number of hits with the appropriate tissue distribution was 432 (Fig. 1E, Supplemental Table 1). In parallel fashion, two methods were undertaken to select a total of 100 genes of highest interest for further study (Fig. 2). One approach was to rank the target genes of interest solely on the maximum positive fold induction of ApoA-I secretion (n=50). The second approach was to curate a list of target genes of interest based on a composite of factors including factors such as existence of genetic associations to HDL/ApoA-I levels in human, phenotype associations in knock-out mice, pathway associations and multiplicity of independent hits in a given pathway, literature text mining, and druggability. After collating the list of genes, additional literature-based research was conducted to determine which genes of interest were tractable from a drug development perspective. Specifically, whether compound inhibitors or knock-out mice that reduce the gene products of interest were available was determined. Based on reagent availability, 59 genes of interest were subjected to deconvolution analysis by studying 4 individual siRNAs from the Smartpool reagents (Fig. 2). Of those genes tested, 40 genes of interest were confirmed regulators of ApoA-I secretion by at least 2 individual siRNAs. The list of the top 40 genes that were confirmed as ApoA-I secretion regulators included multiple cathepsin family members, ion transporters, proteins of unknown function, and metabolic enzymes. The results of mean fold change, Z-score, and sequences of individual siRNA sequences for the confirmed 40 genes are shown in Table 1.
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Repression of farnesyltransferase mRNA expression and activity increases ApoA-I secretion selectively: Inhibition of farnesyltransferase (FNTA) by chemical inhibitors, such as Manumycin A and Zarnesta, demonstrated cardiovascular benefits in preclinical models of disease (9); therefore, we were interested in determining whether ApoA-I levels could be influenced selectively, as compared to other apolipoproteins, by repression of FNTA mRNA and enzymatic activity in cells and in mice. As evaluated in the primary screen, HepG2 cells were transfected with 4 independent siRNAs and the original Smartpool that target FNTA mRNA (Fig. 3A). FNTA mRNA levels were significantly reduced by transfection of the Smartpool and individual siRNAs targeting FNTA mRNA. Likewise, FNTA protein levels were reduced in cells transfected with all siRNA reagents as compared to the non-targeting control protein, GAPDH (Fig. 3B). Transfection of HepG2 cells with the FNTA Smartpool increased ApoA-I secretion by 2.5-fold, whereas, three of four individual siRNAs that comprise the Smartpool caused a statistically significant increase in ApoA-I secretion, although each to a lesser degree as compared to the pooled siRNA reagent (Fig. 3C). These data confirm that FNTA mRNA and protein reduction leads to increased ApoA-I secretion from HepG2 cells. We reasoned that changes to a cell that lead to increased secretion of ApoA-I may likewise lead to increased secretion of other apolipoproteins. Apolipoprotein B (ApoB) is the primary apolipoprotein associated with low density lipoprotein cholesterol (LDL-c) particles, and some evidence suggests that levels of ApoB are the most potent markers of cardiovascular events (10). To evaluate the effect of FNTA mRNA knock-down on ApoB secretion, mass spectrometry measurement of both ApoA-I and ApoB in culture media of HepG2 cells transfected with the FNTA Smartpool and siRNA-1 was conducted. In response to both siRNA treatments, ApoA-I secretion into the media was increased by greater than 2.5-fold as compared to NTC siRNA-transfected cells. By contrast, ApoB secretion was not statistically different from NTC siRNA-transfected cells (Fig. 3D). A profile of higher ApoA-I:ApoB ratio is indicative of improvement in metabolic disease endpoints such as cardiovascular outcomes, diabetic retinopathy, and other sequelae of metabolic syndrome; therefore, a therapeutic molecule with this pharmacology may lead to improved outcomes in patients.

We next determined whether overexpression of FNTA would cause a reciprocal decrease in ApoA-I secretion. Using a mammalian expression vector for human FNTA transfected into HepG2 cells, levels of FNTA were increased over mock transfected cells, and ApoA-I secretion into the medium was decreased by 3-fold as compared to ApoA-I secretion from mock transfected cells (Fig. 3E). These “gain-of-function” data substantiate a role for FNTA in controlling secretion of ApoA-I.

To test whether inhibition of FNTA enzymatic activity would also promote ApoA-I secretion, HepG2 cells were treated with a dose response of a chemical inhibitor of farnesyltransferase, Manumycin A. Manumycin A treatment dose-dependently increased ApoA-I secretion by up to 40% of untreated cells (Fig. 4). These results indicate that both reduction in expression and enzymatic activity of FNTA lead to increased secretion of ApoA-I from hepatocytes.

Lipoprotein profiles in mice differ from human in that expression of the mouse ApoA-I gene is regulated in a manner different from human and human expresses cholesterol ester transfer protein (CETP), but mice do not. Accordingly, the majority of cholesterol in mice circulates as HDL-c particles; whereas, LDL-c particles predominate in human. To assess the ability of Manumycin A to increase circulating levels of human ApoA-I in a preclinical model, we treated mice harboring both a human ApoAI genomic fragment that includes a liver-specific enhancer and the entire human ApoA-I structural gene and the human CETP gene. Manumycin A was given to mice three times per week for two weeks, and human ApoA-I levels were measured from serum on days 1 and 14 post-dose initiation (Fig. 5A). As compared to mice treated with vehicle, Manumycin A caused a statistically significant 26% increase in hApoA-I levels as early as 24 hours post-dose. After 14 days of treatment, hApoA-I levels remained elevated 30% by Manumycin A treatment as compared to vehicle-treated mice (Fig. 5B).
DISCUSSION

The statin class of therapeutics has been successful in reducing LDL-c and cardiovascular risk. However, significant residual risk remains in patient populations; therefore, addressing low HDL-c as a causal risk factor is a promising approach to reduce incidence of cardiovascular outcomes. HDL-c is atheroprotective because it possesses multiple properties—such as anti-thrombotic, anti-inflammatory, anti-oxidant, and its promotion of reverse cholesterol transport from the peripheral tissues including atherosclerotic plaques—that improve such disease promoting mechanisms. In this study, we leveraged a functional genomics approach to find molecular targets for the development of drugs that could elevate ApoA-I, the major protein constituent of HDL-c. The genome-wide functional siRNA screen for increased ApoA-I secretion from HepG2 cells identified numerous candidate gene targets that may be pursued as therapeutic drug targets.

Since the field of GWAS, exome sequencing and whole genome sequencing is rapidly emerging to provide human genetic variant associations with lipid traits important to cardiovascular disease, we assessed whether our genes of interest were also identified by human genetic studies. The seminal study conducted by Teslovich et al. associated common Single Nucleotide Polymorphisms (SNP) with lipid traits in over 100,000 individuals (11). We merged our genes of interest list with the 95 loci for blood lipids that were identified in the Teslovich study and rank-ordered the results based on ApoA-I secretion effect. As expected, the ApoA-I gene itself is a determinant of HDL-c levels in humans (+16.95 effect size, p=7x10^{-240}) and a determinant of ApoA-I secretion in our assay whereby siRNA targeting of the gene resulted in 88% reduction in secreted ApoA-I. Other genes with functional congruence include MC4R, encoding melocortin 4 receptor, whereby an MC4R SNP associates with a -0.42 effect size, p=7x10^{-8}, and MC4R siRNA causes a 1.82-fold increase in ApoA-I secretion, as well as PLTP, encoding phospholipid transfer protein, whereby a PLTP SNP associates with a -0.93 effect size, p=2x10^{-22}, and PLTP siRNA causes a 1.85-fold increase in ApoA-I secretion. None of the genes that were identified in our siRNA screen that were also identified in the GWAS study met the cut-off criteria of Z-score > 2 or < -2. A Supplemental table of loci associated with the HDL-c trait and siRNA targets that modulated ApoA-I secretion has been provided (Supplemental Table 2).

The genes of interest that garnered the most interest were those that modulated ApoA-I secretion by the highest magnitude since those genes may serve either as direct molecular targets for therapeutics or as pathway participants that may highlight druggable pathways for HDL modulation. We reasoned that the functional siRNA screen may identify gene products that could regulate transcriptional activity of the ApoA-I gene, ApoA-I translation efficiency, the rate of secretory flux, or the proteolysis of ApoA-I. Indeed, several gene products with such hypothesized activity that may also be modulated by therapeutics, such as the Cathepsin family of proteases, were identified in the screen (Table 1).

The hypothesis that Cathepsins may directly degrade ApoA-I protein is substantiated by previous reports showing that Cathepsins F and K proteolytic activity can partially degrade lipid-free ApoA-I thereby reducing its ability to induce cholesterol efflux from macrophages. Cathepsin S was shown to completely degrade ApoA-I, leading to complete loss of ApoA-I cholesterol acceptor function (12). Repression of both Cathepsin K and S by siRNA knock-down in our screen resulted in elevation of ApoA-I levels in the HepG2 culture medium, presumably by reducing degradation of ApoA-I.

Another gene target that warranted follow-up was farnesyltransferase or FNTA. Repression of FNTA mRNA by siRNA Smartpool and individual siRNAs resulted in significant increase in secreted ApoA-I from HepG2 cells (Fig. 3C). Notably, the profile of apolipoprotein secretion was favorable because ApoA-I levels, but not ApoB levels, were increased (Fig. 3D). ApoB is the major protein constituent of LDL-c, and one expression of cardiovascular health and risk that is gaining importance in diagnostic medicine is the ApoA-I:ApoB ratio. In the INTERHEART study of cardiovascular risk factors in patients from 52 countries, the non-fasting ApoB/ApoA-I ratio was superior to any of the cholesterol ratios for estimation of the risk of acute myocardial infarction in all ethnic groups, in both sexes, and at all ages (13). Knock-down of FNTA by siRNA specifically raises ApoA-I levels, suggesting its...
potential as target for reducing cardiovascular risk that warrants further follow-up by a variety of methods.

FNTA has been a well-studied molecular target for anti-cancer therapeutics since FNTA-mediated isoprenylation of Ras proteins is necessary for their transforming activities (14). Several small molecules, such as lonafarnib and tipifarnib, have been tested in pre-clinical and clinical studies as cancer therapeutics. Another FNTA inhibitor, Manumycin A, has been studied in a preclinical model of atherosclerosis. When the high fat-fed ApoE null mouse that is highly prone to atherosclerosis was treated with Manumycin A for 22 weeks, fatty streak lesion burden and the area of vascular smooth muscle-like cells in the aortic neointima were reduced (9). When Manumycin A was administered in our HepG2 cell system, ApoA-I secretion levels increased in a dose-responsive manner (Fig. 4).

We reflected on our overall data set and the enzymatic activity of FNTA to generate a hypothesis to describe the mechanisms by which FNTA could be involved in ApoA-I secretion. Generally, maintaining intracellular lipid homeostasis requires that sterols and associated lipids move between cellular compartments by vesicular and non-vesicular pathways. FNTA post-translationally adds a farnesyl group to the -SH of the cysteine near the end of proteins, typically targeting the modified protein to membranes, based on the hydrophobicity of the farneyl group. Centromeric protein F (CENPF) is a farnesylated protein (15), and the siRNA directed toward CENPF caused a 1.98X increase in ApoA-I secretion in our study. We hypothesize that this initial farnesylation event is important for downstream events necessary for appropriate ApoA-I secretion (Fig. 6). This hypothesis is consistent with a study describing CENPF direct interaction with syntaxin 4 revealing a role for CENPF in vesicular transport. Indeed, depletion of cells of CENPF altered GLUT4-mediated glucose transfer (16).

A second alternative pathway that begins with CENPF is also plausible for explaining our observations. CENPF interacts directly with Ndel1, a cytoplasmic dynein binding protein that regulates bi-directional vesicle transport (17,18). In our study, siRNA directed toward Ndel1 increased ApoA-I secretion by 2.29-fold. These data suggest that the coordinated expression and/or subcellular localization either of CENPF or Ndel1 may be necessary for controlling ApoA-I secretion rates. Through functional interactions with other proteins such as CDC42 and dynein, Ndel1 indirectly influences tubulin (TUBB1) and RAB3B. Both proteins are required for vesicular transport and exocytosis, and in our siRNA screen, knockdown of TUBB1 and RAB3B caused a 75% and 74% reduction in ApoA-I secretion. Interestingly, RAB3B is prenylated and also could be a direct target of FNTA (19). In total, our data support a hypothesis whereby FNTA orchestrates protein activities that direct intracellular trafficking of ApoA-I, leading to overall modulation of secreted ApoA-I levels. These findings should be verified in the context of a polarized cell like that of a functional primary hepatocyte. Since these studies were conducted in HepG2 cells that are not polarized, the interactions between these proposed proteins may be real; yet, they may interact differently in a polarized cell setting.

To translate the cell-based findings into a preclinical model of human lipoprotein metabolism, we selected mice that express both human ApoA-I and CETP transgenes for studying in vivo effects of Manumycin A. Following one day of treatment with Manumycin A, circulating levels of human ApoA-I were increased in the double transgenic mice, and the elevated human ApoA-I levels persisted for the 14-day treatment course (Fig. 6). These data illustrate similar regulation of human ApoA-I in both cell culture and in vivo.

Interventions that increase HDL-c by reducing its catabolism in the circulation through inhibition of CETP activity are in late-stage clinical development; however, an alternative approach such as enhancing de novo production of HDL-c in the liver and/or small intestine may provide even greater therapeutic benefit. The anticipated successful reduction of cardiovascular events attributed to elevated HDL-c via CETP inhibition is based on findings from the DEFINE cardiovascular safety trial of anacetrapib (20). In the 1623 patient trial of 76 weeks duration, treatment with anacetrapib, as compared to placebo, increased HDL-c levels by 138.1%, decreased LDL-c levels by 39.8%, and decreased non-HDL-c levels by 31.7%. Importantly, only
2.0% of subjects receiving anacetrapib suffered prespecified, adjudicated cardiovascular safety endpoints as compared to 2.6% receiving placebo. While the data are not conclusive in determining the likelihood of overall reduction in cardiovascular events in longer-term trials, the data suggest potential outcome benefits. Similarly, infusion of recombinant ApoA-I or ApoA-I peptide mimetics are under development and in some studies regression of atherosclerotic plaque area has been observed by intravascular ultrasound measurement (4); however, no direct findings of outcome benefits are available (21).

With increasing insight into emerging qualitative factors that influence HDL functionality with respect to anti-inflammatory properties, capacity to promote reverse cholesterol transport, antithrombotic activity, whether any of the current HDL-elevating strategies will be beneficial remains to be determined in large, outcome-based clinical trials. The results of our study provide new functional genomics-based data for exploring new hypotheses and mechanisms by which ApoA-I levels may be regulated.
References


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FOOTNOTES

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2The abbreviations used are: LDL-c, low density lipoprotein-cholesterol; HDL-c, high density lipoprotein-cholesterol; ApoA-I, Apolipoprotein AI; FNTA, farnesyltransferase; CAD, coronary artery disease; MI, myocardial infarction; NIDDM, non-insulin dependent diabetes mellitus; CETP, Cholesterol Ester Transfer Protein; ABCA-1, adenosine triphosphate-binding cassette protein A-1; LCAT, Lecithin-Cholesterol Acyltransferase; NTC, non-targeting control; HTS, high throughput screening; ADRA1A, Adrenergic Receptor Alpha 1A

FIGURE LEGENDS

**FIGURE 1.** Genome-wide screen for modulation of ApoA-I secretion from HepG2 cells. A, HepG2 cells were transiently transfected with siRNA directed toward human APOA1 as positive control or a non-targeting control (NTC) siRNA that served as a reference for 100% expression of APOA1 mRNA. Cells were cultured for 72 hours, and culture media were collected during the final 24 hours for measurement of ApoA-1 protein by ELISA. Repression of APOA1 mRNA was confirmed by QPCR. Data are presented as the mean ± SEM. Statistical analysis using one-way ANOVA, followed by comparison with NTC by Dunnett’s method, was performed (*p<0.01). B, HepG2 cells were transiently transfected with siRNA directed toward human ADRA1 as positive control or a non-targeting control (NTC) siRNA as a reference for 100% expression of ADRA1 mRNA. Cells were cultured for 72 hours, and culture media were collected during the final 24 hours for measurement of ApoA-1 protein by ELISA. Repression of ADRA1 mRNA was confirmed by QPCR, and repression of ADRA1 protein was determined by western blot analysis. GAPDH protein levels served as a loading control. Data are presented as the mean ± SEM. Statistical analysis using one-way ANOVA, followed by comparison with NTC by Dunnett’s method, was performed (*p<0.01). C, Three plate, three day replication studies were conducted using siRNA targeting either ADRA1 to serve as a positive control for ApoA-1 protein secretion (maximum signal,
Genome-wide screen for ApoA-I secretion

circles), ApoA-1 as a control for reduction of ApoA-1 protein secretion (minimum signal, diamonds), or the NTC to represent no change in ApoA-1 protein secretion (middle signal, plus symbols). Individual datapoints are represented to illustrate the variance and reproducibility of the experimental conditions. D, Human siRNA libraries targeting 21,789 genes were transfected into HepG2 cells to identify genes that are involved in production of ApoA-1 protein. The data are represented as Z-score on the y-axis, and the number of siRNA targets are represented on the x-axis. E, Testing scheme for selecting genes of interest from the whole genome screen illustrates that of 21,789 target genes assessed, 432 genes were identified for follow-up. Gene class categories including the number of genes that may regulate ApoA-1 secretion are listed.

FIGURE 2. Informatic triage strategy. Two methods were undertaken to select a total of 100 genes of highest interest: Ranking target genes of interest based on the maximum positive or negative fold induction of ApoA-I secretion (n=50) or curating genes of interest based on existence of genetic associations to HDL/ApoA-I levels in human, phenotype associations in knock-out mice, pathway associations and multiplicity of independent hits in a given pathway, literature text mining, and druggability. After collating the list of genes, additional literature-based research was conducted to determine which genes of interest were tractable from a drug development perspective (n=59). Individual siRNAs to each gene of interest was tested for the ability to modulate ApoA-1 protein, and 40 genes were confirmed as active.

TABLE 1. List of genes of interest that regulate ApoA-I secretion and are verified by independent siRNA sequences. Mean fold change in ApoA-I secretion following transfection of the Dharmacon Smartpool siRNA for the GOI, the number of individual siRNAs out of four targeting the GOI that modulate ApoA-I secretion, and the sequences of the individual siRNAs that confirmed GOI involvement in ApoA-I secretion are listed.

FIGURE3. In vitro confirmation of FNTA as modulator of ApoA1 secretion. A, HepG2 cells were transiently transfected with either Smartpool or individual siRNAs directed toward human FNTA, and efficiency of mRNA knock-down was determined by Q-PCR. Cells were cultured for 72 hours prior to RNA isolation and Q-PCR. Data are presented as the mean ± SEM. Statistical analysis using one-way ANOVA, followed by comparison with vehicle by Dunnett’s method, was performed (*p<0.01). B, HepG2 cells were transiently transfected with either Smartpool or individual siRNAs directed toward human FNTA, and efficiency of protein knock-down was determined by western blot analysis of FNTA and GAPDH as a protein loading control. C, Specific knockdown of FNTA leads to increase in ApoA-1 secretion from HepG2 cells that were transiently transfected with siRNA directed toward human APOA1 as positive control or a non-targeting control (NTC) siRNA as a reference for 100% expression of APOA1 mRNA. Cells were cultured for 72 hours, and culture media were collected during the final 24 hours for measurement of ApoA-1 protein by ELISA. Data are presented as the mean ± SEM. Statistical analysis using one-way ANOVA, followed by comparison with vehicle by Dunnett’s method, was performed (*p<0.01). D, Mass spectrometry measurement of both ApoA-I and ApoB in culture media of HepG2 cells transfected with the FNTA Smartpool and siRNA-1 was conducted. Normalized peak area for each peptide was converted as percentage of that of the same peptide in the non-transfected cell control, and the average value of the two peptides for the same protein was reported along with percent difference between the two peptides. (*p<0.01). E, HepG2 cells were transiently transfected with either reagent alone (mock) or with a cDNA expression construct for human FNTA, and efficiency of overexpression was determined by western blot analysis of FNTA and GAPDH as a protein loading control. F, HepG2 cells overexpressing human FNTA have a 3-fold reduction in ApoA-I secretion.

FIGURE 4. Dose-dependent inhibition of FNTA by manumycin A increases ApoA1 secretion. Confluent cultures of HepG2 cells were treated with a dose-response of manumycin A for 4 hours prior to ApoA-1 ELISA measurement from the culture media. Data are represented as percent change (mean ±
Genome-wide screen for ApoA-I secretion

SEM) from cells treated with vehicle. Statistical analysis using one-way ANOVA, followed by comparison with vehicle by Dunnett’s method, was performed (*p<0.01).

FIGURE 5. Farnesyltransferase inhibitor, manumycin A, regulates circulating ApoA-I in hApoA-I/hCETP mice. A, Schematic representation of administration of manumycin A to hApoA-I x hCETP transgenic mice. Mice were dosed either with vehicle or 5 mg/kg body weight of manumycin A subcutaneously 3 times per week for two weeks (n=6 mice per group). At day 1 and day 14, blood samples were obtained to measure serum hApoA-I levels by ELISA. Average ApoA-I levels for each group are shown (n=6, ±S.E.M.). Statistical analysis using one-way ANOVA, followed by comparison with vehicle by Dunnett’s method, was performed. (*p<0.01).

FIGURE 6. Mechanistic hypothesis by which FNTA regulates ApoA-1 secretion. FNTA post-translationally adds a farnesyl group (F) to the -SH of the cysteine near the end of proteins, typically targeting the modified protein to membranes, based on the hydrophobicity of the farneyl group. Centromeric protein F (CENPF) is a farnesylated protein, and the siRNA directed toward CENPF caused a 1.98X increase in ApoA-1 secretion in our study. We hypothesize that this initial farnesylation event is important for downstream events necessary for appropriate ApoA-1 secretion. Through indirect (dotted arrows) and direct (solid arrows) interactions with downstream proteins known to be involved in vesicle transport and exocytosis, FNTA directs downstream events that lead to ApoA-1 secretion.
Figure 1

A

% expression

0
25
50
75
100
125

ApoA-I mRNA
ApoA-I protein

NTC
ApoA-I siRNA

* *

B

% expression

0
25
50
75
100
125
150
175

ADRA1 mRNA
Secreted ApoA-I

NTC
ADRA1 siRNA

* *

C

Fold Over NTC

0
0.5
1
1.5
2
2.5

0 Day1/Plt
Day1/Plt2
Day1/Plt3
Day2/Plt
Day2/Plt2
Day2/Plt3
Day3/Plt
Day3/Plt2
Day3/Plt3

Days/Plate
Figure 1

D

Z-score

siRNA targets (21,789)

E

Human Genome libraries:
- GPCR
- Kinase
- Druggable
- Rest of Genome (ROG)
- 21,789 total targets

Human Genome
- 756 hits
- Z-score $\pm 2$

Rescreen of Hits With SMART pools
- 85% confirmation rate
- 432 hits

Liver/Intestine Expression Filter
- GNF Affymetrix >300
- OncoGenomics ratio > 0.3
- 432 hits
Figure 2

**Biology**
- Molecular network/pathways
- Disease/Phenotype association
- GWAS from dbGaP (database of Genes and Phenotypes)
- Expression correlation
- Proteome MESH Disease Classification
- HuGE Gene Prospector
- Gene Ontology Classification
- Mouse Phenotype Classification

**Absolute regulation**
- Magnitude of ApoA-I induction or repression

- Prioritize genes based on availability of pharmacology tools
- 40 out of 59 hits confirmed by siRNA deconvolution
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**Table 1**

Genome-wide screen for ApoA-I secretion
Figure 3

A

B

C

D

Genome-wide screen for ApoA-I secretion
Figure 3

E

Mock  FNTA cDNA

FNTA

GAPDH

F

% expression

Mock  FNTA Over Exp

Genome-wide screen for ApoA-I secretion
Figure 4
Figure 5

A

Day: 0 2 5 7 10 12

Dose am Dose pm Dose am Dose pm Dose am Dose pm

bleed bleed

B

ApoA-I (ng/ml ± SEM)

Vehicle Manumycin A

Day 1 Day 14

* *
Figure 6

Genome-wide screen for ApoA-I secretion

- **CENPF**
  - siRNA ↑ A-I 1.98

- **FNTA**
  - siRNA ↑ A-I 2.5

- **Syntaxin 4**
  - Vesicular transport

- **NDEL1**
  - siRNA ↑ A-I 2.29

- **TUBB1**
  - siRNA ↓ A-I 0.254

- **RAB3B**
  - siRNA ↓ A-I 0.26

- **ApoA-I**
Genome-wide screen for modulation of hepatic Apolipoprotein A-I (ApoA-I) secretion
Rebecca R. Miles, William Perry, Joseph V. Haas, Marian K. Mosior, Mathias N'Cho, Jian
W.J. Wang, Peng Yu, John Calley, Yong Yue, Quincy Carter, Bomie Han, Patricia
Foxworthy, Mark C. Kowala, Timothy P. Ryan, Patricia J. Solenberg and Laura F. Michael
J. Biol. Chem. published online January 15, 2013

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