

A synonymous variant in scavenger receptor, class B, type I gene is associated with lower SR-BI protein expression and function

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ABSTRACT

Objective: A synonymous variant within scavenger receptor class B type I gene (*SCARB1*), exon 8 rs5888, has been associated with altered lipid levels and cardiovascular risk in humans. The objective was to determine if rs5888 decreased SR-BI protein expression and function *in vitro*.

Methods: SR-BI RNA secondary structure, turnover, polysomal distribution and protein expression were examined in COS cells transfected with wild-type or rs5888-SR-BI plasmids by selective 2'-hydroxyl acylation and primer extension assays, actinomycin D inhibition, polysomal profiling, and western blotting. SR-BI function in murine macrophages stably expressing wild-type or rs5888-SR-BI was assessed by measuring the specific cell association of ¹²⁵I,³H-cholesteryl ester (CE) radiolabeled HDL.

Results: Rs5888 changed RNA secondary structure and led to marked differences in the polysomal profiles compared with wild-type transcript ($p < 0.02$). As compared to wild-type cells, COS cells expressing rs5888 had significantly lower SR-BI protein expression ($p < 0.04$), but no difference in total RNA transcript levels. There were no differences in SR-BI RNA turnover in murine macrophages, whereas specific cell association of ¹²⁵I ($p < 0.0001$) or ³H-CE ($p < 0.00001$) was significantly lower in rs5888 cells.

Conclusions: The rs5888 variant affected SR-BI RNA secondary structure, protein translation, and was significantly associated with reduced SR-BI protein expression and function *in vitro*.

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1. Introduction

While much is known regarding the role of scavenger receptor class B type I (SR-BI) in cardiovascular and reproductive physiology in mice [1], much less is known about its role in humans. This receptor was first isolated and characterized as a physiologically relevant lipoprotein receptor in murine tissues by Acton et al. [2]. The receptor is highly expressed in the liver, and in steroidogenic tissues such as the adrenal glands and gonads [3]. SR-BI participates in the selective uptake of cholesteryl esters (CE) from HDL, low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL); it is regulated by a number of factors including corticotrophin, estrogens, cyclic AMP, gonadotropins, testosterone and peroxisomal proliferator activated receptors [4–8].

Acton et al. [9] were the first to identify single nucleotide polymorphisms (SNPs) of the *SCARB1* gene in a white European population and associated some of these common variants with plasma lipid levels and body mass index (BMI). The nonsynonymous exon 1 SNP (rs4238001 [G2S], which encodes for an amino acid change) was significantly associated with higher HDL cholesterol (HDL-C) and lower LDL cholesterol (LDL-C) levels. The synonymous or silent exon 8 SNP (rs5888 [A350], which does not encode for an amino acid change) was associated with lower LDL-C levels, and the intron 5 SNP (C>T) showed an association with BMI. These authors were the first to suggest that *SCARB1* SNPs might affect lipid metabolism in humans.

We recently reported a significant association between the rs5888 SNP and lipid levels in a cohort of the Amish Family Diabetes Study [10]. We found that HDL-C levels were significantly higher in women carriers of the exon 8 SNP, suggesting an association of this synonymous SNP with lower SR-BI protein levels. Our findings were consistent with Richard et al. [11], wherein these investigators had also previously reported an interaction with the exon 8 SNP and increased HDL-C levels.

Until recently, synonymous or silent SNPs were thought to be only markers for unidentified causal SNPs; however, accumulating

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evidence now suggests that synonymous SNPs might directly affect protein expression and/or function of their gene products [12–14]. For example, Kimchi-Sarfaty et al. [15] showed that a synonymous SNP of the Multidrug Resistance 1 (*MDR1*) gene affected the conformation of its gene product, P-glycoprotein, and consequently the functionality of this protein.

Given the results of our previous work showing a significant association between the rs5888 SNP and HDL-C levels in humans [10], the goal of the present study was to determine if the synonymous *SCARB1* rs5888 SNP might directly affect SR-BI protein expression and function. Using several *in vitro* models, we indeed found that the rs5888 SNP affected SR-BI RNA secondary structure, which changed its ability to undergo productive protein translation leading ultimately to significantly lower SR-BI protein expression. Moreover, SR-BI function was significantly lower in macrophages expressing the rs5888 variant.

2. Methods

2.1. Prediction of RNA secondary structure

Secondary structures of both wild-type and rs5888-SR-BI full-length cDNA were predicted using the *Mfold* program (version 3.2). Transcript sequences were folded in an automated manner by the *Mfold* program (<http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi>) to generate secondary structures.

2.2. Generation of human SR-BI expression vectors

See online supplement.

2.3. Transient transfection of COS-7 cells

See online supplement.

2.4. Stably expressing wild-type and rs5888 murine RAW macrophage cell lines

See online supplement.

2.5. Cell association uptake assays.

See online supplement.

2.6. mRNA turnover assays

See online supplement.

2.7. Western blotting

See online supplement.

2.8. Polysomal profiling experiments

COS cells (2×10^7 cells) were transfected with either 200 ng/ml of DNA containing pSG5-wild-type or rs5888 using FuGene6 according to the manufacturer's protocol. Polysomal RNA was isolated as previously described [19]. Briefly, immediately prior to sample collection the cells were exposed to 100 μ g/ml cycloheximide for 15 min at 37 °C. Cells were washed in 4 °C Dulbecco's PBS containing cycloheximide. Inclusion of cycloheximide fixed ribosomes along a strand of RNA, thus eliminating ribosomal run-off and stabilizing the polysomal RNA. Cells were lysed in low salt buffer (LSB, 20 mM Tris (pH 7.4), 10 mM NaCl and 3 mM MgCl₂) containing 1.2% Triton N-101. Cells were pelleted and resuspended

in ~400 μ l of polysomal lysis buffer supplemented with Rnasin (Promega) and a cocktail of proteinase inhibitors. The cells were homogenized in an ice-cold glass dounce homogenizer and 3/4 of the supernatant was transferred into a tube containing 100 μ l of LSB containing heparin (10 μ g/ μ l). The remaining 1/4 of lysate sample representing total RNA was placed in Tri Reagent (Sigma). The supernatant was layered onto a continuous sucrose gradient (15–40%) and centrifuged at 36,000 \times g for 4 h. Continuous gradients were made using the Thermo Gradient Maker. Following centrifugation, each sucrose/RNA gradient was pushed through the Thermo UA6 optical detector (254 nm) with integrated chart recorder and fraction collector in order to identify the fractions corresponding to the non-polysomal, 80S ribosomal, and polysomal RNA as previously described [19]. RNA from individual fractions were then isolated and analyzed by quantitative RT-PCR. Each experiment was performed three times.

2.9. Selective 2'-hydroxyl acylation and primer extension (SHAPE) assays [20,21]

DNA encoding exons 7–9 of wild-type and rs5888-SR-BI RNA were generated by PCR from plasmid templates and this product was used in *in vitro* transcriptions, which were purified by denaturing gel electrophoresis, passively eluted into RNase-free water, and concentrated using Amicon concentrators. Four picomoles of pure RNA was renatured by heating to 85 °C for 45 s and cooled on the bench top, and then 3 μ l of folding buffer [333 mM Hepes (pH8.0), 333 mM NaCl, 20 mM MgCl₂] was added to the RNA (final volume of 30 μ l) and incubated at 37 °C for 10 min. To probe the RNA, either 1 μ l of 130 mM *N*-methylisatoic anhydride (NMIA) in anhydrous dimethylsulfoxide (DMSO), or DMSO alone was added to the RNA and reactions were incubated 37 °C for 60 min, then passed through a Micro Bio-Spin® P-30 column. Modified positions were mapped by annealing ~2 pmol of modified RNA to a 5'-[³²P]-labeled cDNA primer and conducting reverse transcription (RT) using SuperScript™ Reverse Transcriptase III (Invitrogen). RT reactions were resolved on 10% denaturing polyacrylamide sequencing gels (alongside dideoxy-sequencing lanes), dried, visualized using a phosphorimager, and analyzed using the programs ImageQuant, Excel, and KaledaGraph. Each experiment was performed twice.

2.10. Native gel electrophoresis

Two micrograms of wild-type and rs5888 RNAs (exons 7–9) were incubated with either 2.5 mM MgCl₂ or 2.5 mM EDTA in 10% glycerol + 1 \times TH buffer (66 mM Tris, 34 mM HEPES, pH 7.4) at 37 °C for 5 min (20 μ l final volume). Samples were run on either 8% polyacrylamide gels (1 mm thick) in 1 \times TH buffer + either 2.5 mM MgCl₂ or 2.5 mM EDTA at 5 W each, 4 °C for 16 h. Gels were removed from the apparatus and visualized with ethidium bromide.

2.11. Statistical analysis

Student's *t*-test was used for the analyses. Two-way ANOVA was used to determine statistical differences in the polysomal experiments. Probability values less than 0.05 were considered statistically significant.

3. Results

Given that rs5888 is a common (minor allele frequency ~40%) synonymous or silent SNP residing within exon 8 of the *SCARB1* gene, our first step was to examine whether the minor allele might predict changes in the SR-BI mRNA secondary structure. As shown in online supplement Fig. 1, using *Mfold* software [22], substitution

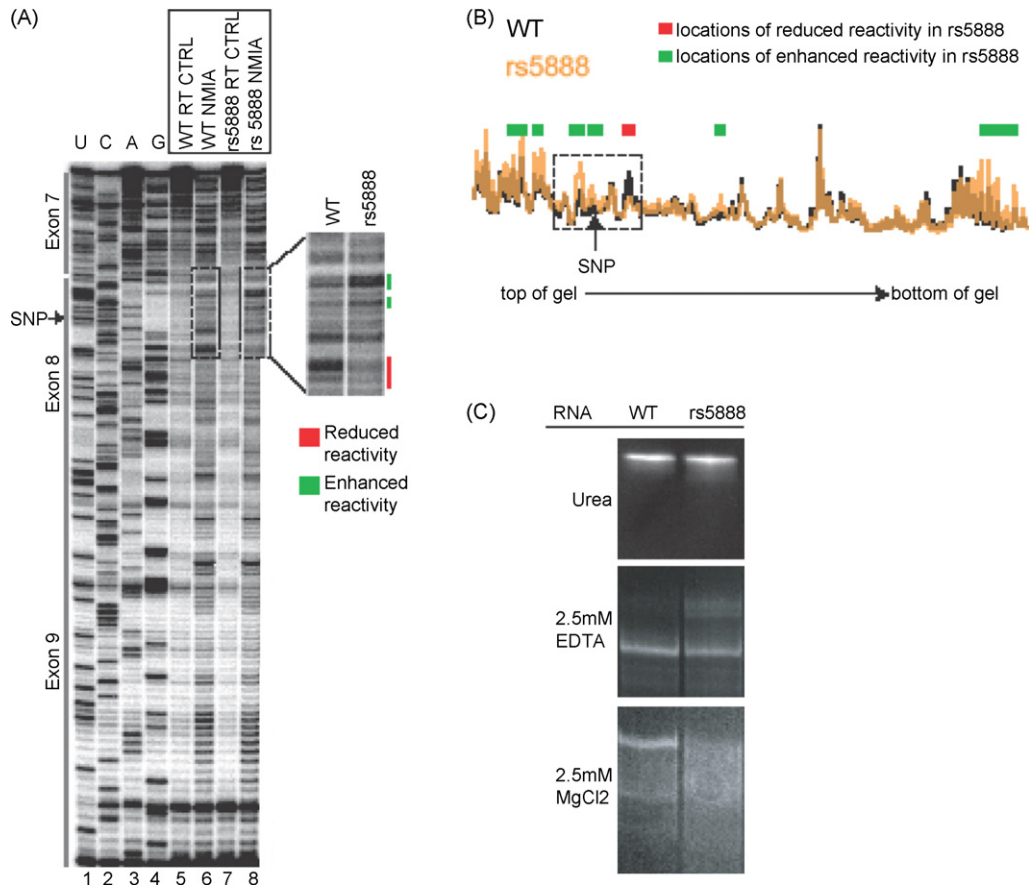


Fig. 1. RNA structural changes due to SNP. (A) Gel of wild-type and rs5888 RNAs probed using SHAPE, oriented 5' at the top and 3' at the bottom. Exons 7–8 are indicated by vertical grey line, and the C → T SNP indicated by an arrow. Lanes 1–4 are dideoxy-sequencing ladders, lanes 5 and 7 contain control reactions for dimethyl sulfoxide (DMSO) background cleavage, and lanes 6 and 8 contain the NMIA modified wild-type and rs5888 RNAs. Dashed boxes and enlarged gel region highlights a region of interest where rs5888 is becoming more single-stranded (green) or more double stranded (red) as compared to wild-type. (B) A quantitated trace of the gel shown in panel (A), and turned on its side. Black is WT and Orange is rs5888. The two are overlaid. The dashed box indicates the portion of the gel that is boxed in panel A. The two traces are very similar, showing that overall, the secondary structures between the two are quite similar. However, distinct areas of local changes are seen. These are indicated with red or green bars above the trace. There is a cluster of changes right around the site of the SNP. (C) Native gel analysis of both wild-type and rs5888 RNAs in 2.5 mM EDTA (top panel, unfolded) or 2.5 mM MgCl₂ (bottom panel, folded). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of the major C allele with the minor T allele predicted changes in the secondary structure of the SR-BI mRNA.

Thermodynamic-based models predicted that the rs5888 variant would lead to an alteration of the mRNA secondary structure in the vicinity of the SNP. To directly test this prediction, we next used selective 2' acetylation monitored by primer extension (SHAPE) to probe the secondary structure of *in vitro* transcribed and purified RNA comprising exons 7–9 of both wild-type and rs5888-SR-BI. In the SHAPE secondary structure probing assay, modification of the 2' hydroxyl by NMIA depends on whether the nucleotide is base paired (2'-OH protected) or single-stranded (2'-OH modified), and hence changes in the modification pattern indicate changes in secondary structure. When the modification patterns of wild-type and rs5888 exons 7–9 RNAs are compared (Fig. 1A and B), specific differences are observed, indicating a change in secondary structure. The most clearly observed differences are in the vicinity of the variant and these are clearly visible on the gel (Fig. 1A), but other regions of subtle difference are found in other parts of exons 7–9 (Fig. 1B). To further explore our observation that rs5888 possessed an altered structure, we subjected the exons 7–9 RNA to native gel electrophoresis. Native gel electrophoresis is very sensitive to changes in the overall global structure of RNA; these changes are reflected in a change in the migration rate of the altered RNA relative to wild-type RNA. When *in vitro* transcribed and purified wild-type and rs5888 RNA were electrophoresed through a gel containing 2.5 mM EDTA and a gel containing 2.5 mM MgCl₂, differences in the migra-

tion patterns were observed (Fig. 1C). In the EDTA gel, the RNA is not folded into its full native structure, but residual secondary structure can form. In EDTA, the wild-type RNA runs as a single band, whereas the rs5888 RNA has a second, lower mobility band visible. This pattern likely indicates that alternate secondary structures can form in the variant RNA that are not formed in the wild-type RNA. In the Mg²⁺-containing gel, RNA forms its native structure. In Mg²⁺ wild-type RNA runs as two bands, with the upper band being more intense. However, the rs5888 RNA runs as a smeared band, suggesting a change in the conformation of the RNA that may be interpreted as a structurally heterogeneous population with exchange between different conformational states. Note that these differences are not due to differences in the purities of the two RNA samples, as both run as a single tight band on a denaturing gel (Fig. 1C, uppermost urea band).

To test whether the rs5888 SNP was associated with problems with mRNA translation, we performed polysomal profiling experiments to probe for alterations in SR-BI protein translation. Polysomal RNA was isolated from transiently transfected COS cells expressing the full-length wild-type or rs5888-SR-BI transcripts. As shown in Fig. 2A, total SR-BI RNA levels were not significantly different between wild-type and rs5888 expressing cells. However, there was a significant difference between the overall distribution of SR-BI RNA transcripts in wild-type expressing cells compared with rs5888 cells (Fig. 2B, *p* < 0.02, two-way ANOVA). Specifically, in the wild-type expressing cells, the majority of the SR-BI transcripts

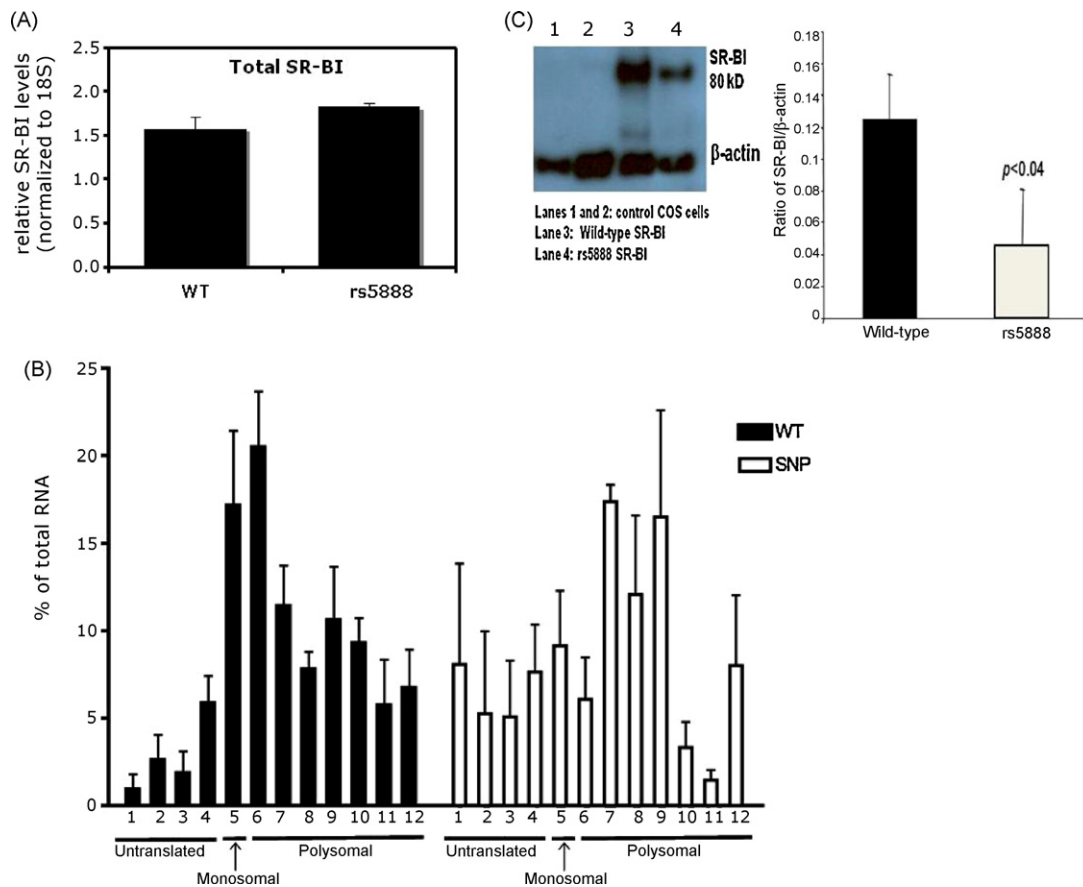


Fig. 2. Polysomal profiling of SR-BI mRNA in transfected COS cells. (A) SR-BI RNA levels were similar in cells transfected with either wild-type and rs5888-SR-BI plasmids. (B) Polysomal profiling showed that the rs5888-SR-BI does not properly assemble ribosomal units resulting in increased amounts of SR-BI mRNA associated with the RNP, 40S and 60S pools (i.e., fractions 1–5) when compared to the wild-type ($p < 0.02$, two-way ANOVA), and (C) SR-BI protein levels were significantly lower in COS cells transfected with rs5888-SR-BI compared with cells transfected with wild-type SR-BI, $p < 0.04$ compared to wild-type cells. The western blot image is representative of three independent experiments, while the bar graphs are the mean \pm standard error of three independent experiments.

migrated with the monosomal and disomic fractions (fractions 5 and 6), with the remainder associated with almost equal levels of the heavier polysomal fractions (fractions 7–12). Conversely, the results for the rs5888 expressing cells showed a shift in SR-BI RNA transcripts to the lighter fractions (i.e., non-translating fractions; fractions 1–3 contain the ribonucleoprotein associated, 40S, and 60S associated mRNAs). Additionally, the rs5888-SR-BI transcripts were associated with polysomal fractions (fractions 7–9). The mRNA transcripts in these fractions contain 4–6 polysomes, respectively. Moreover, as shown in Fig. 2C, SR-BI protein levels were significantly lower in rs5888 expressing cells compared with wild-type cells (67% lower, $p < 0.04$).

Given that total RNA transcripts were similar between wild-type and rs5888-SR-BI expressing cells but not the distribution of the two transcripts in the polysomal fractions, we next determined whether these effects might be due to alterations in SR-BI RNA turnover. We examined SR-BI RNA turnover in murine macrophages stably expressing wild-type and rs5888-SR-BI in the presence of actinomycin D (20 $\mu\text{g}/\text{ml}$) for varying periods of time (0–24 h). As shown in Fig. 3, baseline total SR-BI RNA levels were similar in wild-type and rs5888 expressing macrophages. More importantly, in the presence of actinomycin D, 18S and SR-BI RNA turnover were similar between the wild-type and rs5888-SR-BI macrophages, suggesting that differences in SR-BI protein expression were not due to an effect of the variant on RNA transcription.

We then examined whether expression of rs5888 would affect SR-BI function. Nontransfected, wild-type and rs5888-SR-BI expressing macrophages were exposed to ^{125}I , ^3H -CE labeled HDL

(5 μg protein/ml) in the presence and absence of excess, unlabeled HDL (250 μg protein/ml) for 16 h at 37 $^{\circ}\text{C}$. As shown in Fig. 4, specific cell association of the radiolabeled apoprotein moiety (^{125}I) ($p < 0.0001$) and of the lipid labeled moiety (^3H -CE) ($p < 0.00001$) were significantly lower in rs5888 expressing cells as compared with wild-type cells. Selective CE uptake (ng/mg cell protein) was

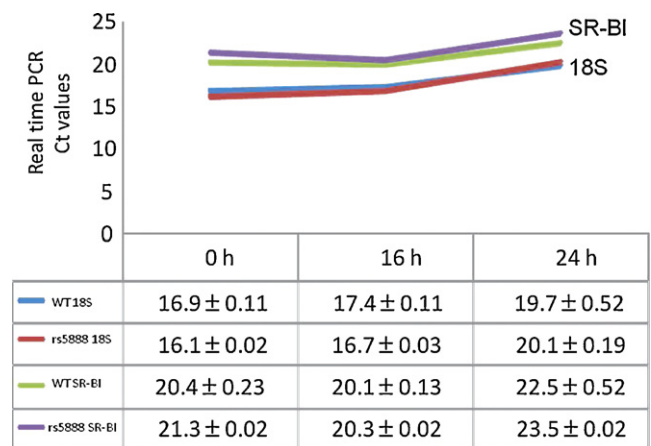


Fig. 3. SR-BI mRNA turnover is similar between macrophages stably expressing wild-type and rs5888-SR-BI. SR-BI and 18S RNA levels were similar in wild-type and rs5888-SR-BI expressing macrophages in the presence of actinomycin D (20 $\mu\text{g}/\text{ml}$) for varying periods of time (0–24 h).

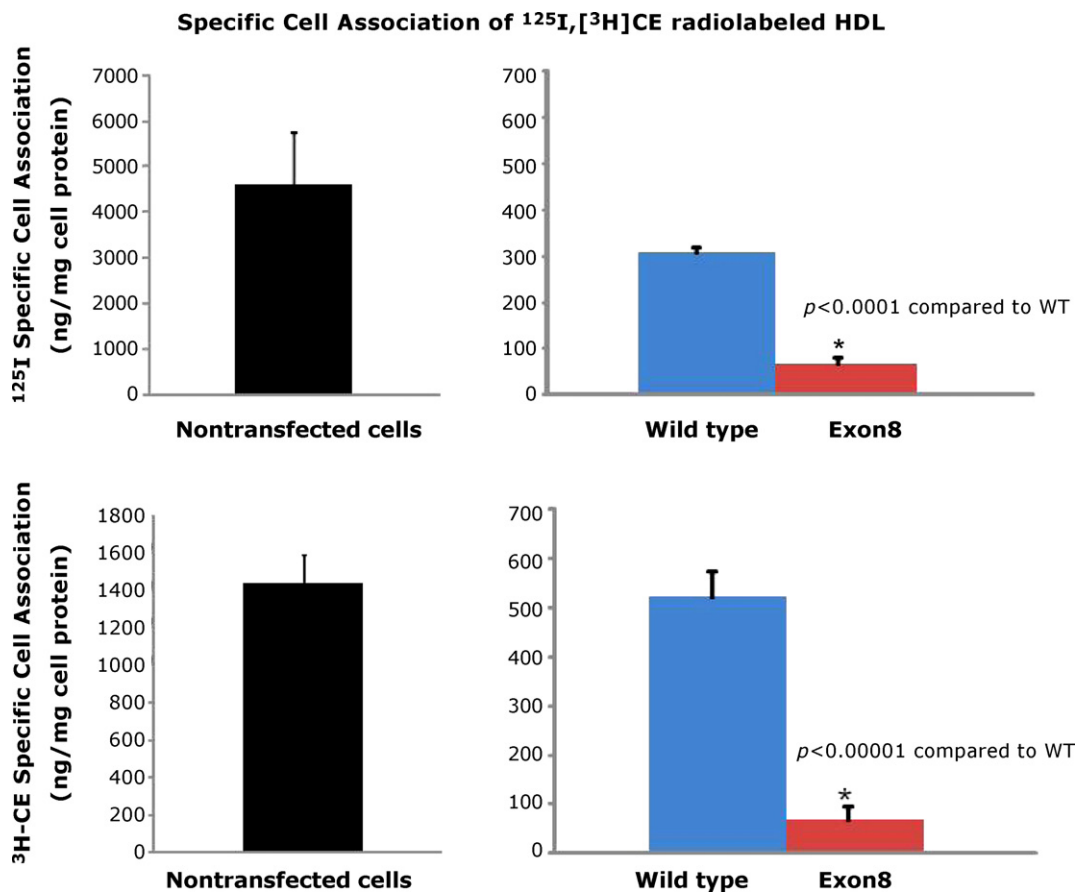


Fig. 4. Specific cell association of ^{125}I , ^3H -CE labeled HDL is significantly lower in murine macrophages stably expressing rs5888-SR-BI. Nontransfected, wild-type, and rs5888 stably expressing RAW macrophages were incubated with ^{125}I , ^3H -CE labeled HDL (5 μg protein/ml) in the presence and absence of excess, unlabeled HDL (250 μg protein/ml) for 16 h at 37 °C. Specific cell association of the ^{125}I -labeled apoprotein moiety ($p < 0.0001$) and of the ^3H -CE labeled moiety ($p < 0.00001$) were significantly lower in cells expressing the rs5888-SR-BI compared to wild-type cells. The results are the mean \pm standard error of two independent experiments.

also calculated as per Kraemer et al. [23] and the results showed negative selective CE uptake for nontransfected cells (or more holoparticle uptake), 1.2 ng/mg cell protein for rs5888 cells, and 214 ng/mg cell protein for wild-type SR-BI expressing cells.

4. Discussion

That a synonymous SNP altered the prediction of the mRNA secondary structure was consistent with observations reported by Nackley et al. [24] and others [25]. Nackley et al. [24] found that synonymous haplotypes of the catechol-O-methyltransferase gene (*COMT*) also predicted changes in the mRNA secondary structure, based on predictions generated by the *Mfold* software. These synonymous SNPs predicted structures with lower free energies and with configurations that were more stem-like, similar to what we have observed for the synonymous rs5888 SNP.

The mechanism by which synonymous SNPs change the secondary structure is unclear. It is possible that changes in the secondary structure interfered with initiation of protein synthesis at the AUG start site. It is possible that the altered secondary structure interferes with loading of the ribosomal 40S and 60S subunits on to the mRNA, as this is suggested by the results of the polysomal profiling, wherein SR-BI RNA levels are elevated in the untranslated fractions isolated from cells transfected with the rs5888 plasmid.

The lack of change in mRNA expression showed that the reduced SR-BI protein expression by the rs5888 SNP was due to a post-transcriptional effect. The results of the polysomal profiling showed an enrichment of the rs5888 RNA in untranslated fractions. To the

best of our knowledge, there have not been other publications that specifically examined the effect of synonymous SNPs on protein translation using polysomal profiling. However, Signori et al. [26] did utilize polysomal profiling to examine the effects of a somatic mutation in the 5'-UTR of *BRCA1* gene on translation efficiency. These investigators transiently transfected HEK293 cells with plasmids containing either the wild-type or mutant plasmids and then performed polysomal profiling. They found that cells transfected with the mutant allele had mRNA that was more associated with the lighter, monosomic fractions, suggesting a defect in protein translation. We also do not believe that the post-transcriptional effects of the rs5888 variant on SR-BI protein expression is Dicer miRNA/siRNA dependent, as Dicer has been shown to be undetectable in monocytes and virtually undetectable or absolutely undetectable in terminally differentiated macrophages [27]. We also confirmed this observation by Klase et al. [27] in that we were unable to detect Dicer protein expression in either wild-type or rs5888 macrophages transfected with scrambled or Dicer-siRNA oligonucleotides (data not shown).

We are the first to show that a synonymous *SCARB1* SNP significantly reduced SR-BI function *in vitro* as evidenced by the significant reduction in specific cell association of dual-labeled HDL. Our findings are consistent with those of Acton et al. [2] and many subsequent investigators [28–30] who have shown strong positive correlations between SR-BI protein expression and activity. Nonetheless, more work is needed to fully understand the intracellular processing and cell surface functionality of rs5888-SR-BI alone, or as part of haplotypes, associated with and without cardiovascular disease. The results from the study by Duan et al.

[25] suggested that the biological effect of a particular SNP might be greatly influenced by its association with other SNPs in a haplotype. Our studies concentrated solely on the effect of the rs5888 SNP alone, and not on its effect within a haplotype.

While SR-BI knockout mice, in the background of either apoE or LDL receptor deficiency, have been shown to have accelerated atherosclerosis [1], little is known regarding complete SR-BI deficiency in humans. We were the first to show that deficiency of SR-BI in primary human macrophages is inversely correlated with HDL levels [31], but we have yet to identify subjects with complete SR-BI deficiency. While it is tempting to speculate that human carriers of the rs5888 variant might be SR-BI deficient (especially since we [10] and others [9,11] have shown associations of rs5888 with higher HDL levels and lower SR-BI protein levels), our study [31] lacked sufficient power to determine a significant association between SR-BI protein levels in human carriers of the rs5888 variant and measures of atherosclerotic disease. Nonetheless, we are continuing to recruit subjects with the aim of elucidating whether this variant or others, either alone or as part of a haplotype, significantly affect SR-BI protein expression, and in turn, affect risk for cardiovascular disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2009.11.029.

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