Comparing expression and activity of PCSK9 in SPRET/EiJ and C57BL/6j mouse strains shows lack of correlation with plasma cholesterol☆

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Abstract

Objective: Low-density lipoprotein receptor (LDLR) and proprotein convertase subtilisin/kexin type 9 (PCSK9) are opposing regulators of plasma LDL-cholesterol levels. The PCSK9 gene exhibits many single or compound polymorphisms within or among mammalian species. This is case between the SPRET/EiJ (SPRET) and C57BL/6j (B6) mouse strains. We examined whether these polymorphisms could be associated with differential expression and activity of their respective PCSK9 molecules.

Methods: Liver expression of LDLR and PCSK9 transcripts were assessed by RT-PCR, and that of their corresponding proteins by immunoblotting. Purified recombinant PCSK9 proteins were assayed for their ability to degrade LDLR. Pcsk9 gene proximal promoters were tested for activation of a luciferase reporter gene.

Results: SPRET and B6 mice carried comparable levels of plasma cholesterol in spite of the fact that SPRET mice expressed less PCSK9 and more LDLR in liver. There were indels and single-base differences between their Pcsk9 cDNA and promoter sequences. Ex vivo, SPRET Pcsk9 protein was less secreted but was more active at degrading LDLR. Its gene promoter was more active at driving expression of the luciferase reporter.

Conclusions: Collectively, these results suggest that, compared to the B6 mouse, the SPRET mouse may represent an example of absence of direct correlation between PCSK9 and cholesterol levels in plasma, due to genetic variations leading to reduced secretion of PCSK9 associated with greater LDLR-degrading activity.

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1. Introduction

Cholesterol circulates in blood plasma as lipoprotein particles, primarily as high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very low-density lipoproteins (VLDL). LDL is the main cholesterol transporter in humans as HDL is in mouse [1]. The plasma level of LDL-cholesterol (LDL-C) is determined in large part by the level of their receptors (LDLR) at the surface of hepatocytes. Normally, LDLR binds LDL-C from blood, takes it into the cell and cycles back to the cell surface [2]. The recycling is down regulated by proprotein convertase subtilisin/kexin type 9 (PCSK9), a self-inactivated enzyme turned escort protein, which is also predominantly produced by hepatocytes. PCSK9 binds to LDLR at the cell surface, gets endocytosed with it, prevents its recycling to the surface, rerouting it to lysosomes where it is degraded [3]. High level or activity of PCSK9 reduces the amount of LDLR at the surface of hepatocytes, resulting in diminished clearance of blood LDL-C; and vice versa. This has been widely confirmed by genetic epidemiology in humans [4–9], and by genetic or physiological manipulations in mice [10–12].

A phylogenetic study has shown that, apart from a region in the catalytic domain, most of the vertebrate PCSK9 sequence can tolerate amino acid substitutions [13]. Moreover, the coding sequence of the human PCSK9 gene contains numerous non-synonymous single-nucleotide polymorphisms (SNPs). With regards to the LDLR-degradation activity of the variant protein, some SNPs lead to a gain of function (GOF); others lead to a loss of function (LOF); others yet are neutral. GOF SNPs are associated with an increase of circulating cholesterol; LOF SNPs with its decrease [4,9,14]. Multiple SNPs of diverging phenotypes may sometimes be carried by the same Pcsk9 allele [9]. In such cases of compound polymorphism, a measure of the level of circulating LDL-C combined with ex vivo evaluation LDLR-degrading activity of the polymorphic protein should help clarify the resultant phenotype. We decided to test this approach in mice. While exploring the Mouse Phenome Database established by The Jackson Laboratory (phenome.jax.org) for differences among inbred strains in plasma level non-HDL-C using the C57BL/6J (B6) mouse as reference, we noticed that the Mus spretus SPRET/EiJ (SPRET) mouse contained the least. Here we investigate

☆ The sequences of the cDNA and the gene proximal promoter of SPRET/EiJ mouse Pcsk9 have been deposited in the GenBank database under the accession numbers KX818098 and KX818097, respectively.

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whether the difference in plasma non-HDL-C level between the two strains was associated with corresponding difference in expression of PCSK9 and its LDLR-degrading activity.

2. Materials and methods

2.1. Materials

B6 and SPRET mice were maintained at the Jackson Laboratory (Bar Harbor, ME) in quiet rooms at about 20 °C and 50% humidity with 12-h light/dark cycle. They were provided with water and food at libitum. The food, LabDiet®5K0G, provided 22% of calories from protein, 16% from fat and 62% from carbohydrates. It contained 0.240% (g/g) of cholesterol. Plasma and liver from 8-weeks old male mice were collected following an approved necropsy protocol. The Jackson Laboratory is an institution assured by the Office of Laboratory Animal Welfare (OLAW) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACi); for the care and use of laboratory animals, it abides by the guidelines of the National Institute of Health. Human hepatocytes Huh7 and human embryonic kidney HEK293 cells were obtained from Dr. Nabil G. Seidah (Clinical Research Institute of Montreal).

2.2. Reagents

The following reagents were from commercial sources: goat anti-mouse LDLR, horseradish peroxidase (HRP)-conjugated anti-mouse PCSK9 antibodies (Ab), and PCSK9 enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems; mouse anti-β-actin Ab, and β-actin Ab, HRP-conjugated sheep anti-mouse IgG antibody at 1:10,000 dilution and HRP-conjugated sheep anti-mouse IgG secondary antibody at a 1:10,000 dilution. The densitometric values of β-actin bands were used for normalization of experimental samples.

2.3. Lipid assays

Colorimetric assays for triglycerides, total cholesterol as well as HDL-C and LDL-C/VLDL-C (hereafter called LDL-C) were performed using kits from BioVision; RNeasy extraction kit. It was reverse-transcribed into cDNA using oligo-dT primers and the Superscript II RNase H⁻ Reverse Transcriptase. The levels of PCSK9, LDLR and TATA-binding protein (TBP) cDNAs were quantified by PCR-based fluorogenic Taqman assays [15], using FastStart TaqMan Probe Master-Rox master mix, primer pairs and the appropriate fluorescent UPL probes (Supplementary Table 1S), in a Mx3005P thermocycler (Stratagene). Standard curves were established using varying amounts of purified and quantified cDNA amplicons of each mRNA. The level of TBP mRNA was used for normalization.

2.4. Cell culture

Huh7 or HEK293 cells were normally cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for maintenance or LPDS for experiments, and 50 μg/ml gentamycin at 37 °C under a humidified 5% CO₂-95% air atmosphere. After experimental incubations, spent media were centrifuged at 200 g for 5 min to sediment suspended cells; supernatants were collected and supplemented with 0.02 volumes of a 50 × stock PIC; as needed, cell monolayers were rinsed with ice-cold phosphate-buffered saline (PBS), overlaid with radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate and 0.1% SDS) supplemented with 1 × PIC, and placed on an ice bath for 20 min; lysates were centrifuged at 14000 g for 4 °C for 20 min, and supernatants collected. Spent media of and cell lysates were stored at −20 °C until processing or analysis.

2.5. qRT-PCR

Total RNA was extracted using the Qiagen RNeasy extraction kit. It was reverse-transcribed into cDNA using oligo-dT primers and the Transcriptor II RNase H⁻ Reverse Transcriptase. PCR primers, HRP-conjugated anti-V5 tag Ab, and Lipofectamine Reagent Plus from Invitrogen; FastStart TaqMan ProbeMaster-Rox master mix, Universal Probe Library (UPL) fluorescent probes, and the Protease Inhibitor Cocktail (PIC) from Roche.

2.6. Immunoblotting

Cell lysates were fractionated by SDS-PAGE and electrophoretically transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with a goat anti-mouse LDLR (at a dilution of 1:15000) and then with a HRP-conjugated heterospecific secondary anti-goat Ab (1:5000). Alternatively, the membrane was treated directly with HRP-conjugated anti-mouse PCSK9 Ab. In all cases, it was probed for HRP reaction using the chemiluminescence-based revelation kit. The signal was captured on X-ray film and immunoreactive bands analyzed by densitometry on a Syngene’s ChemiGenius²XE Bio Imaging System within the dynamic range of the instrument. The membrane was stripped and re-probed with the anti-β-actin monoclonal primary antibody at 1:10,000 dilution and HRP-conjugated sheep anti-mouse IgG secondary antibody at a 1:10,000 dilution. The densitometric values of β-actin bands were used for normalization of experimental samples.

2.7. Purification of recombinant PCSK9

The open reading frame (ORF) of B6 and SPRET mouse Pcsk9 cDNA with V5 tag-encoding extension was subcloned into pCIneo under the human cytomegalovirus promoter-enhancer, to produce the pCIneo-B6/Pcsk9-v5 and pCIneo-Spret/Pcsk9-v5 expression vectors, respectively. Alternatively, the V5-encoding sequence in the above vectors was replaced by a double stranded oligonucleotide encoding an hexahistidyl peptide to generate the pCIneo-B6/Pcsk9-H6 and pCIneo-Spret/Pcsk9-H6 expression vectors, respectively. The latter vectors were transiently transfected into human embryonic kidney HEK293 cells in a medium containing 2% FBS; spent media were collected and supplemented with 1 mM PMSF; Pcsk9-H6 was purified from these media by affinity chromatography using Qiagen Ni-NTA agarose as recommended by the manufacturer. The accuracy of the subcloned cDNAs was verified by sequencing; the purity of the protein by SDS-PAGE followed by silver staining. The protein was quantified by ELISA.

2.8. Promoter activity assays

The proximal promoter region (~600-base pair upstream to the initiator ATG) was amplified from B6 and SPRET genomic DNA and subcloned into the pGL4.13-luc2 vector, upstream to the luciferase gene, to generate pGL4-B6/Pcsk9Pr-Luc2 and pGL4-Spret/Pcsk9Pr-Luc2 reporter vectors. Triplicate wells of Huh7 cells (7.5 × 10⁵/well) were each transfected with 1 μg of either vector using Lipofectamine Reagent Plus; after 48 h, cell lysates were prepared and their protein content determined by Bradford dye assay; the luciferase activity of 5 μl of lysate was measured on a Promega luminometer using a Luciferase Assay System as recommended by the same manufacturer.

2.9. Statistical analysis

The number of mice (n) per strain was 8–9. All experiments were conducted at least three times, each in triplicates. Means and standard errors were computed. Statistical analyses were performed using the GraphPad Prism software, version 6.
3. Results

3.1. Plasma LDL-C levels are comparable between SPRET and B6 mice despite a relative deficit of PCSK9 in SPRET liver and plasma

The plasma cholesterol profile of fed SPRET and B6 male mice are described in Fig. 1. Compared to the B6 mouse, the SPRET mouse showed a trend towards higher levels of total cholesterol (TC) (Fig. 1Aa, \(P = 0.055\)), carried significantly higher levels of HDL-C (Fig. 1Ab, \(P = 0.012\)) (Fig. 1A), but similar levels of LDL-C (Fig. 1B, \(P = 0.295\)), leading to significantly lower LDL-C/HDL-C ratio (Fig. 1C, \(P < 0.001\)). Triglyceride (TG) levels were similar between the two strains (Fig. 1D).

Because plasma levels of lipoprotein-cholesterol depend on hepatic balance between PCSK9 and LDLR, expression of these two molecules in SPRET and B6 liver was examined. Protein levels were measured by immunoblotting and mRNA levels by RT-PCR. Compared to B6 livers, SPRET livers contained 10-fold less PCSK9 (Fig. 2Aa, \(P < 0.001\)) and 1.25-fold more LDLR (Fig. 2Ab, \(P < 0.01\)). They contained 2.3-fold less of PCSK9 transcript (Fig. 2Ba, \(P < 0.0001\)) (Fig. 2B), but similar amounts of LDLR transcripts. Consistent with the level of its transcript in the liver, plasma PCSK9 in the SPRET mouse was about half that in the B6 mouse (Fig. 2C, \(P < 0.0001\)).

3.2. SPRET and B6 PCSK9 exhibit multiple amino acid differences

The PCSK9 full-length cDNAs of SPRET and B6 mice were amplified by RT-PCR from liver transcripts and sequenced. The amino acid sequences deduced from their open reading frames (ORF) were aligned for comparison. Compared to B6 sequence, SPRET sequence included a L-L-P16–18 deletion in the signal peptide, as well as the non-synonymous differences A302T, R305Q, T408A, T477I, W502Q, V532L, H556Y, R577Q, R578K, A581V, and A665D (Supplementary Fig. 1S). SIFT analysis (http://sift.bii.a-star.edu.sg) predicted that all the amino acid differences between SPRET and B6 PCSK9 were tolerable. PolyPhen-2 analysis (http://genetics.bwh.harvard.edu), on the other hand, concluded that the H556Y variation was possibly damaging i.e. could alter the LDLR-degrading activity of the protein.

3.3. SPRET PCSK9 is less secreted than its B6 homolog

To assess whether, taken globally, these differences in amino acids could result in differences in protein expression or secretion the ORFs of the cDNA were cloned into an expression vector and transiently transfected into the Huh7 hepatocyte line. PCSK9 levels in cell lysates and culture media were measured by ELISA. Relative to that of B6, SPRET PCSK9 accumulated more inside the cells (Fig. 3A, a, \(P = 0.018\)), and was less secreted (Fig. 3A, b, \(P < 0.001\)). This intracellular accumulation was confirmed by immunoblotting (Fig. 3B, a, \(P < 0.005\)). As a result of the transfection, the level of endogenous LDLR in Huh7 cells was reduced, confirming the activity of the induced proteins (Fig. 3B, b).

3.4. SPRET PCSK9 degrades LDLR more efficiently than its B6 homolog

The efficiency of the two murine PCSK9 at degrading LDLR was compared by exposing the same number of cells to increasing amounts of the proteins supplemented to the culture media. These results are shown in Fig. 4. Under the experimental conditions, at 0.5, 1, and 2.5 \(\mu\)g per well, SPRET PCSK9 reduced cellular LDLR level by 32, 51, and 67%, respectively; whereas B6 PCSK9 reduced it by 12, 15, and 42%, respectively. For both proteins, the reduction reaches its nadir at 5 \(\mu\)g per ml. These results indicate that SPRET PCSK9 is more efficient at degrading LDLR than its B6 homolog, presumably because of the differences identified in their primary structures.
3.5. The promoter of SPRET Pcsk9 gene is more active than its B6 homolog

The steady state level of Pcsk9 mRNA in the liver is lower in SPRET mice compared to B6 mice (see Fig. 2B). Possible alternative explanations include reduced transcription of its gene or increased degradation of its mRNA. To verify the first possibility, the 700 bp of the Pcsk9 gene upstream to the translation start codon was PCR amplified from genomic DNA in both strains of mice and sequenced. Fig. 5 shows putative high affinity binding sites identified therein by Transcription Factor Affinity Prediction (TRAP) algorithm [http://trap.molgen.mpg.de]. They include binding sites for Nescient Helix Loop Helix 1 ([NLH1], Hepatocyte Nuclear Factor 4c (HNF4A), Spermatogenic Leucine Zipper 1 (SP1), E74-Like Factor 4 (ELF4), NHF 1c (HNF1A) and Sterol-Regulated Element Binding Protein (SREBP), Activator Protein 2 (AP-2), Deleted In Esophageal Cancer 1 (DEC1), and Hypermethylated In Cancer 1 (HIC1). Except for the latter site, they are all conserved between the two strains.

The promoters were subcloned into an expression vector upstream to the cDNA of a luciferase reporter. The vectors were transfected into HuH7 hepatocytes and expression measured. The results showed that the Pcsk9 proximal promoter from the SPRET mouse was 2.2-fold more active than that from B6 (Fig. 6, *P < 0.001), suggesting that sequence differences between the two promoters reduced or enhanced the activity of one relative to the other.

4. Discussion

4.1. The SPRET LDL-C paradox

Experimental evidence in mice has indicated a direct correlation between the level of Pcsk9 and that of total cholesterol as well as lipoprotein-cholesterol in plasma [10,12,16,17]. When compared to the B6 mouse, the SPRET mouse exhibits the paradoxical situation of a trend towards higher plasma TC in the presence of lower plasma Pcsk9. The TC trend was primarily due to HDL-C as the LDL-C levels were comparable between the two strains. The lower level of plasma Pcsk9 (2-fold) was associated with lower hepatic content of Pcsk9 transcript (10-fold), suggesting a difference in mRNA production or stability. The higher hepatic content in LDL-C (2.5-fold) would be consistent with the lower plasma level of Pcsk9 (i.e. reduced Pcsk9-mediated degradation of the receptor), was it not for the absence of any significant difference in plasma LDL-C. A possible explanation for the conundrum could be a relative inefficiency of the SPRET LDLR at clearing this lipoprotein. This has not been examined in this study. Also not examined in the two mouse strains was the influence of fibroblast growth factor 21 (FGF-21) cascade which is known to upregulate the level of cellular LDLR [18].

Another study comparing the plasma lipid profiles of B6 and SPRET mice, 7 to 9 weeks of age, found that females of both strains showed similar levels of non-HDL-C, but male SPRET mice had significantly less than their B6 counterparts (*P = 0.011) [19,20]. It should be noted that the mice in that study were fasted for 4 h prior to blood collection whereas ours were not. Whether fasting differentially affects the quantification parameters of LDL-C is unknown.

4.2. The combined effects of multiple variations

Differences between the two strains in the primary structure of Pcsk9 include an indel located in the signal peptide and several nonsense variations dispersed along the remainder of the proprotein. A leucine addition in the signal peptide of Pcsk9 has been linked to reduced mean plasma LDL-C in Caucasians [9,21]. The effect of the addition on Pcsk9 biosynthesis and activity has not been determined. Whether Leu-Leu-Pro_18-18 deletion in the signal peptide of SPRET Pcsk9 is responsible for its reduced secretion is unknown at this time. To answer this question, one could construct expression vectors in which signal...
peptides are swapped between B6 and SPRET PCSK9, transfect them into cells and examine their effects on secretion by pulse-chase studies.

Of all the missense variations, only H556Y was estimated to be potentially deleterious by PolyPhen-2 analysis. The H556Y difference is located in the M2 domain of the carboxyl terminal cysteine and histidine-rich domain (CHRD) of PCSK9. The M2 domain appears to be required for LDLR degradation through the extracellular pathway[22]. Moreover, the H556Y variation is located at the same position as the H553R variation found in humans in whom it is associated with high plasma LDL-C[4]. Rather than analyzing the effect of single variations, we chose to examine the overall consequences of all the amino acid differences combined. Collectively, these differences rendered SPRET PCSK9 less efficient at secretion when intracellularly expressed in Huh7 cells (Fig. 4), but more efficient at LDLR degradation when extracellularly added to the culture media of these cells (Fig. 5). Thus the fact that plasma LDL-C is practically similar between the two strains could alternatively be explained also by the greater activity of SPRET PCSK9 partially compensating for its reduced presence in circulation. Interestingly, a bioinformatics analysis could be carried out to identify structural changes associated with cumulative amino acid variations when a high resolution structure of mouse PCSK9 becomes available in the Protein Data Bank (PDB).

Fig. 5. Sequence alignment of B6 and SPRET proximal Pcsk9 gene promoter. Nucleotide differences and indels are shaded. Putative binding sites for transcription factors are underlined and the factors written below with the TRAP weight score in parentheses. The ATG initiating the open-reading frame is doubly underlined.
4.3. More active gene promoter, but less mRNA

The liver is the predominant source of circulating PCSK9 [12]. The lower plasma level of this protein in SPRET mice relative to B6 mice is consistent with decreased level of its mRNA in the liver. The cause of this decreased expression of Pcsk9 transcripts is still to be determined. It cannot be attributed to the SPRET proximal promoter of the gene which, in vitro, was found to be more active than its B6 homolog. The cis elements for HNF1A and SREBP2 known to regulate this promoter [23–25] are conserved between the two strains. Among other putative high affinity binding sites, HIC1 which is present in B6 promoter is of some interest because it is aberrated in SPRET promoter and the difference scored as most significant \((P = 5.1 \times 10^{-5})\) by TRAP comparative analysis (http://www.bitnos.com/transcription-factors-analysis). HIC1 is a widely expressed zinc finger transcriptional repressor that interacts with other factors in repression complexes to regulate cell growth and survival [26]. Although Pcsk9 has not been identified yet among its target genes, the loss of its binding site in the SPRET promoter could tentatively explain its greater activity under our in vitro assay conditions. In vivo on the other hand, it is possible that other variations of cis acting elements located at more distal positions combined with differing abundance of trans acting factors come into play to influence Pcsk9 transcription in the two strains.

A similar argument can be invoked when considering the possibility that SPRET Pcsk9 mRNA, even if produced in greater amounts, might be more prone to degradation. There is a single canonical AU-rich element (ARE) in the 3′-untranslated region (3′-UTR) of mouse Pcsk9 mRNA (TAUUUAAT); it is conserved between the two strains. Its possible contribution to the degradation of this transcript in SPRET mouse hepatocytes implies greater accessibility to ARE-binding proteins mediating this degradation [27]. An alternative possibility involves microRNA (miRNA) regulation of Pcsk9 miRNA abundance through differences in target sequences in their 3′-UTR or in expression level of specific miRNAs. We screened the 3′-UTRs using various online algorithms (e.g., TargetScan, RegRNA, miRDB, or microRNA) and identified several putative targets, miR-1966 being the only one that is present in SPRET but not in B6 Pcsk9 3′-UTR. On the other hand, Timmermans et al. [28] have reported a comparative analysis of basal mRNA and miRNA expression in the liver of the two strains; they identified many differences and validated several mRNA-miRNA interactions. Our examination of their data showed miR-10b to be obviously more expressed in SPRET than in B6 mouse. It remains to be experimentally examined whether miR-1966 target and miR-10b miRNA contribute to the degradation of hepatic Pcsk9 mRNA in SPRET mouse.

4.4. The SPRET mouse vs the PCSK9 knockout mouse?

Although it is now well-established that the PCSK9-LDLR opposing tandem regulates plasma cholesterol, novel implications of this tandem in other physiological pathways are emerging [29], most dramatic among them being the clearance of toxins following bacterial infection [30–32]. There are intriguing phenotypic similarities between the SPRET mouse and the engineered PCSK9 knockout (PCS9KO) mouse: both exhibit glucose intolerance [16,33,34], resistance to endotoxin-induced septic shock [32,33,35], and resistance to high fat-induced atherosclerosis [19,36]. It may be premature to conclude that the partial PCSK9 deficiency observed in the SPRET mouse contributes to these common phenotypes. One way to further this argument is to explore whether the PCS9KO mouse exhibits some other phenotypic traits of the SPRET mouse such as lower levels of total testosterone and resistance to certain bacterial and viral infections as well as to certain forms of cancer [37]. A more cogent proof of the implication of PCSK9 deficiency to these phenotypes in the SPRET mouse would be their reversal through replacement therapy involving chronic intravenous administration of a recombinant form of the protein or its transgene-mediated expression in the liver. More applicable to human biology would be the exploration of these phenotypic traits in a chimeric mouse model of transplanted with human liver [38,39] that carries an inactive PCSK9 gene.

5. Conclusions

Relative to the B6 mouse, the SPRET mouse could represent a model of PCSK9 protein deficiency associated with enhanced LDLR-degrading activity. The dichotomous properties probably result from the sum of variations in its gene. Monoallelic combined PCSK9 variations have been observed in humans too [9]. Our results in the mouse illustrate the need to evaluate them collectively and not individually, as the overall effect of multiple variations on protein conformation and activity could differ from that of single variations.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2016.11.006.

Conflicts of interest statements

None of the authors has a conflict of interest to report.

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