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RNA therapeutics inactivate PCSK9 by inducing a unique intracellular retention form

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ABSTRACT

Hypercholesterolemia is a medical condition often characterized by high levels of low-density lipoprotein cholesterol (LDL-C) in the blood. Despite the available therapies, not all patients show sufficient responses, especially those with very high levels of LDL-C or those with familial hypercholesterolemia. Regulation of plasma cholesterol levels is very complex and several proteins are involved (both receptors and enzymes). From these, the proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged as a promising pharmacologic target.

The objective of this work is to develop a new approach to inactivate PCSK9 by splice-switching oligonucleotides (SSOs), converting the normal splice form to a natural, less abundant and inactive, splice variant.

For this purpose, a new RNA therapeutic approach for hypercholesterolemia based on SSOs was developed for modulation of the splice pattern of human *PCSK9* pre-mRNA. Our results show an increase of the selected splice form at both the mRNA and protein level when compared to non-treated Huh7 and HepG2 cell lines, with concomitant increase of the protein level of the low-density lipoprotein receptor (LDLR) demonstrating the specificity and efficiency of the system. *In vivo*, full conversion to the splice form was achieved in a reporter system when mice were treated with the specific oligonucleotide, thus further indicating the therapeutic potential of the approach.

In conclusion, PCSK9 activity can be modulated by splice-switching through an RNA therapeutic approach. The tuning of the natural active to non-active isoforms represents a physiological way of regulating the cholesterol metabolism, by controlling the amount of LDL receptor available and the rate of LDL-cholesterol clearance.

1. Introduction

Often characterized by high levels of low-density lipoprotein cholesterol (LDL-C) in plasma, hypercholesterolemia [1] is one of the major risk factors for the development of atherosclerosis [2] and cardiovascular disease, principal causes of death in developed countries. Statins, a class of cholesterol lowering drugs [3], are the established therapy for lowering LDL-C levels in patients but, although considered efficient and safe, all of them present some limitation at higher doses [4]. Moreover, statins do not sufficiently reduce LDL-C in high-risk or very high-risk subjects [5], and frequently add-on therapies are required [4].

Several genes and their coded proteins controlling cholesterol metabolism have been studied in order to identify additional pharmaceutical targets. One of the most promising is the proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 was discovered in 2003 [6] and identified as the third locus associated to autosomal dominant hypercholesterolemia [7]. In 2005, a study from K. Maxwell et al. demonstrated that overexpression of PCSK9 mediated by an adenoviral vector accelerates the degradation of the low-density lipoprotein receptor

Abreviations: CTR, control; CRISPR, clustered regularly interspaced short palindromic repeats; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LDL-C, low-density lipoprotein cholesterol; LDLR, low-density lipoprotein receptor; LNA, locked nucleic acid; LPDS, lipoprotein deficient serum; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline Tween®20; PCSK9, proprotein convertase subtilisin/kexin type 9; PCSK9fl, full-length proprotein convertase subtilisin/kexin type 9; PCSK9sv, splice variant proprotein convertase subtilisin/kexin type 9; PMO, phosphorodiamidate morpholino; RIPA, radioimmunoprecipitation assay; RP-HPLC, reverse-phase high-performance liquid chromatography; siRNA, small interfering RNA; SEM, standard error of the mean; SREBPs, sterol-regulatory element binding proteins; SSO, splice-switching oligonucleotides; TBE, Tris/Borate/EDTA; TBST, Tris-buffered saline Tween®20.

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(LDLR) in hepatic cells [8]. The hepatic cell surface LDLR is the major protein responsible for the clearance of the LDL-C from plasma. Since then, several other studies have shown the relation between gain [9] and loss [9–11] of function mutations in *PCSK9* with the corresponding increase and decrease of LDL-C levels in the blood, respectively.

PCSK9 is a natural post-transcriptional inhibitor of LDLR. It binds with high affinity to the LDLR (by the pro-segment/catalytic portion) and induces its internalization and/or degradation in lysosomes [12] through extra and intra [13] cellular pathways, regulating the amount of LDLR at the membrane level. PCSK9 acts like a chaperone protein and its function is independent of its catalytic activity [5]. Decreasing the membrane LDLR levels promote the increase of LDL-C in the blood. Like other genes involved in the metabolism of cholesterol, *PCSK9* is regulated by the transcription factors sterol-regulatory element binding proteins (SREBPs) and thus is dependent on the amount of sterols in the cell [14]. The levels of *PCSK9* are inversely related with the amount of cellular sterols, increasing when depleted and decreasing when accumulated [14].

PCSK9 is mainly expressed in liver, small intestine, kidney and central nervous system [6] and mediates the degradation of LDLR not only in hepatocytes, but also in fibroblasts and macrophages, but not in kidney or adrenals [15]. Regarding other biological functions, genome-wide expression analysis studies predict that *PCSK9* could be involved in the down-regulation of stress-response genes and specific inflammatory cytokine pathways [16,17]. Additionally, an *in vitro* study shows that PCSK9 can reduce the levels of the epithelium sodium channel and so modulate the epithelial sodium absorption, thereby PCSK9 may have a role controlling blood pressure [18].

Therapies to decrease the expression of the PCSK9 protein through down-regulation of its mRNA by antisense oligonucleotides [19–22] or small interfering RNA (siRNA) [23,24]; or inhibit its ability to bind to the LDLR, mediated by antibodies [25,26]; or by disruption of the gene by introduction of loss of function mutations by a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system [27] have been developed in the last years with variable outcomes [5,28].

In 2008, Schmidt et al. [29] described the existence of a PCSK9 splice isoform (PCSK9sv), which is expressed in multiple tissues, but contrary to full-length PCSK9 (PCSK9fl), does not show activity in controlling cellular levels of LDLR protein. PCSK9sv differs from PCSK9fl by an in-frame deletion of exon 8, preventing autocatalytic cleavage of the pro-domain and the secretion of the protein [29]. When compared with the full transcript, this splice form corresponds only to 18%. *In vitro* and *in vivo* studies (in HepG2 cells, human immortalized lymphoblasts and livers from African green monkeys) show that sterol depletion suppresses the relative amount of the alternatively spliced transcript, while addition of LDL-cholesterol conversely augments this fraction [30]. In the same study, the half-life of the two transcripts was investigated, showing that the full-length *PCSK9* transcript has a significantly reduced half-life after sterol depletion, while there was no change in mRNA stability of its alternatively spliced variant.

The finding of a human, natural, non-functional splice form opens a new perspective for targeting intracellular PCSK9 in a more physiological manner. Here we demonstrate that it is possible to modulate the two *PCSK9* splice forms by splice-switching oligonucleotides (SSOs) in two human hepatocyte cell lines, Huh7 and HepG2, and in an *in vivo* context, suggesting that this represents a promising approach for the development of novel therapeutics.

2. Materials and Methods

2.1. Oligonucleotide design

We used a combination of three predictive bioinformatics programs: ESE3 (ESEfinder v3.0) [31,32], PESX and RESCUE-ESE Web Server (v1.0) [33,34] to predict target-accessibility in order to design a rational series of SSOs, each individually targeting exon 8 of the human *PCSK9* gene (GenBank accession no. **NM_174936.3**). Each oligonucleotide sequence was BLASTed against all human sequences deposited in GenBank® databases. SSOs were synthesized as fully modified RNA 2′-*O*-methyl-phosphorothioate oligonucleotides and RP-HPLC purified by DNA Technology A/S (Denmark). After being dissolved in nuclease-free water, the concentration of SSOs was assessed using a NanoDrop (Thermo Scientific) and stored as frozen aliquots at −20 °C.

The commercially available siRNA used in this study is specific for human *PCSK9* (SASI_Hs01_00154292, SIGMA).

2.2. Oligonucleotide transfections

Hepatic HuH7 and HepG2 cells were maintained and cultivated in Dulbecco's Modified Eagle's medium plus 10% FBS at 37 °C, 5% CO_2 and 95% humidity. The cells were seeded at a density of 10^4 (Huh7) and 1.4×10^5 (HepG2) cells per well in a 24-well plate the day before transfection, in order to be approximately 80% confluent the next day. Transfection of the SSO constructs and the siRNA was performed with Lipofectamine® 2000 (Life Technologies) or Lipofectamine® RNAiMax (Life Technologies), respectively, and according to the manufacturer's protocols. The transfection complexes were left in the culture for 24 h, after which medium was changed or cells were harvested.

2.3. Sterol starvation by LPDS incubation

Hepatic Huh7 were seeded at a density of 3×10^4 cells per well in a 6 well plate the day before transfection in order to be approximately 50–60% confluent the next day. Transfection of the SSOs constructs and siRNA was performed as in Subsection 2.2. The complexes were left in the culture for 24 h, after which medium was removed, cells washed with PBS 0.01 M and fresh Dulbecco's Modified Eagle's medium without phenol red (to prevent interference with the method used to measure the concentration of cholesterol) plus 10% LPDS (lipoprotein deficient serum) was added to the cultures to induce sterol starvation. Twenty-four hours later, medium was removed and fresh Dulbecco's Modified Eagle's medium without phenol red plus 10% LPDS supplemented with 20 nmol per well of human LDL-C was added to the cells. Cells were allowed to stay in the cholesterol-supplemented conditions for 12 h, after which cells were washed, and collected for RNA and protein isolation.

2.4. Viability assay

Viability was assayed with the CellTiter-Glo Luminescent cell viability assay (Promega) according to the manufacturer's protocol. Briefly, after 24 h incubation of the cells with the transfection complexes, the cultures were allowed to equilibrate for 30 min at room temperature, after which an equal volume of the CellTiter-Glo reagent was added to the wells. Following cell lysis and luminescence stabilization, values of luminescence were measured.

2.5. RNA expression analysis

Total RNA was isolated with the RNeasy® plus mini kit (QIAGEN) according to the manufacturer's protocol. In the animal experiment, total RNA from livers was isolated with Tri-Reagent® (Sigma-Aldrich) and DNase treated with RapidOut DNA Removal Kit (Thermo Scientific) according to the manufacturer's protocols. Quantity and quality of the total RNA were determined by NanoDrop by analysis of the ratios at 260/280 nm and 260/230 nm.

In the cell work RT-PCR was performed with 10 ng of the isolated RNA in each reaction (total volume per reaction was 20 μL) with the QIAGEN® OneStep RT-PCR kit (QIAGEN) and following the manufacturer's protocol. In the animal experiments, RT-PCR was performed with 25 ng of cDNA in each reaction (total volume per reaction