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PCSK9-deficient mice exhibit impaired glucose tolerance and pancreatic islet abnormalities

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9), a liver-secreted plasma enzyme, restricts hepatic uptake of low-density lipoprotein (LDL) cholesterol by promoting the degradation of LDL receptors (LDLR). PCSK9 and LDLR are also expressed in insulin-producing pancreatic islet β cells, possibly affecting the function of these cells. Here we show that, compared to control mice, PCSK9-null male mice over 4 months of age carried more LDLR and less insulin in their pancreas; they were hypoinsulinemic, hyperglycemic and glucose-intolerant; their islets exhibited signs of malformation, apoptosis and inflammation. Collectively, these observations suggest that PCSK9 may be necessary for the normal function of pancreatic islets.

1. Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) belongs to a 9-member family of serine endoproteinases, structurally related to bacterial subtilisin and to yeast kexin. These enzymes are found in the secretory pathway of all cells, in varying combinations and levels. Collectively, they are responsible for the activation of a wide variety of precursor proteins by endoproteolysis after selected residues [1]. PCSK9 is predominantly produced and secreted by the liver [2,3]. It has no known physiological substrate.

The most studied property of PCSK9 is its ability to down regulate hepatic uptake of low-density lipoprotein-cholesterol (LDL-C) by promoting intracellular degradation of the LDL receptor (LDLR). According to the current prevailing model, the convertase attaches to the receptor and directs it to endosomes/lysosome-like compartments for degradation [4–6]. Inactivation of the *Pcsk9* gene in mouse increases hepatic LDLR content and the clearance of plasma LDL-C, leading to hypocholesterolemia [3,7]. Its overexpression in the liver of transgenic mice has the opposite effect [3,8–12].

Pancreatic β cells express significant amounts of LDLR [13,14]. These receptors can mediate the uptake of exogenous lipoproteins by established β cell lines and isolated islets in culture. Chronic exposure to high LDL or VLDL is lethal for these cells [14,15]. Death occurs through endogenous heavy metal-catalyzed oxidative stress since it can be abrogated by chelating agents and antioxidants [15]. A survey of established cell lines indicated that RIN-m5F and βTC-3 lines derived from insulin-producing pancreatic β cells contained substantial amounts of PCSK9 mRNA [2,3]. We hypothesized that PCSK9 protects these cells from the toxic effects of excessive cholesterol accumulation. Comparing wild type and PCSK9-null mice, we show in this report that male mutant mice exhibit impaired glucose homeostasis.

2. Materials and methods

2.1. Mice

129Sv;C57BL/6 (129;B6) *Pcsk9*$^{+/-}$ male mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were backcrossed with B6 *Pcsk9*$^{+/+}$ females for eight generations. The B6-N8 *Pcsk9*$^{+/-}$ incipient congenic mice were then intercrossed to

Abbreviations: IB, immunoblotting; IP, immunoprecipitation; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; LDLR, LDL receptor; OGTT, oral glucose tolerance test; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; PCSK9, proprotein convertase subtilisin/kexin type 9; sqIB, semi-quantitative immunoblotting; TBP, TATA-box binding protein

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generate *Pcsk9*$^{-/-}$ and *Pcsk9*$^{+/+}$ mice used in these studies at 4–5 months of age. Mice were treated according to guidelines of the Canadian Council on Animal Care under a protocol approved by an institutional Animal Care Committee. They were housed in temperature-controlled (25 °C) rooms with a 12-h light/dark cycle and given access to standard mouse chow and water *ad libitum*, except when overnight fasting (12–16 h) was required.

2.2. Oral glucose tolerance test (OGTT)

Mice were fasted overnight, weighed and blood (~0.05 ml) was collected by submandibular puncture and supplemented with EDTA to a final concentration of 10 mM. They were then fed glucose (1.5 mg/g body weight) by oral gavage and were bled as above after 15, 30, 60 and 120 min. Blood samples were centrifuged for 10 min at 1100×*g* to sediment cells; plasma was collected and stored at −80 °C until analysis.

2.3. Pancreatic islet isolation and culture

Mouse pancreatic islets were isolated following ductal injection of collagenase buffer (1.5 mg/ml) [16,17]. Briefly, mice were anesthetized and sacrificed by cervical dislocation; pancreata were injected with a collagenase solution; islets were handpicked under a microscope.

2.4. Glucose and insulin and lipid assays

Glucose levels were determined using the Beckman Coulter glucose analyzer and insulin levels were measured using the Ultra Sensitive Mouse Insulin ELISA Kit from Crystal Inc. (Downers Grove, IL).

2.5. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The levels of specific mRNAs were quantified in a PCR-based fluorogenic assay using the Taqman technology [18]. Briefly, total RNA was extracted using the RNeasy extraction kit from Qiagen (Mississauga, ON) and reverse-transcribed into cDNA using random hexameric primers and the Superscript II RNase H$^-$ Reverse Transcriptase (Invitrogen). The cDNA was used as a template to produce PCR amplicons using FastStart TaqMan ProbeMaster-Rox master mix, primer pairs and the appropriate fluorescent probe from the Universal Probe Library (UPL) (Roche, Laval, QC) (Supplementary Table 1S) in a Mx3005P thermocycler (Stratagene, LaJolla,

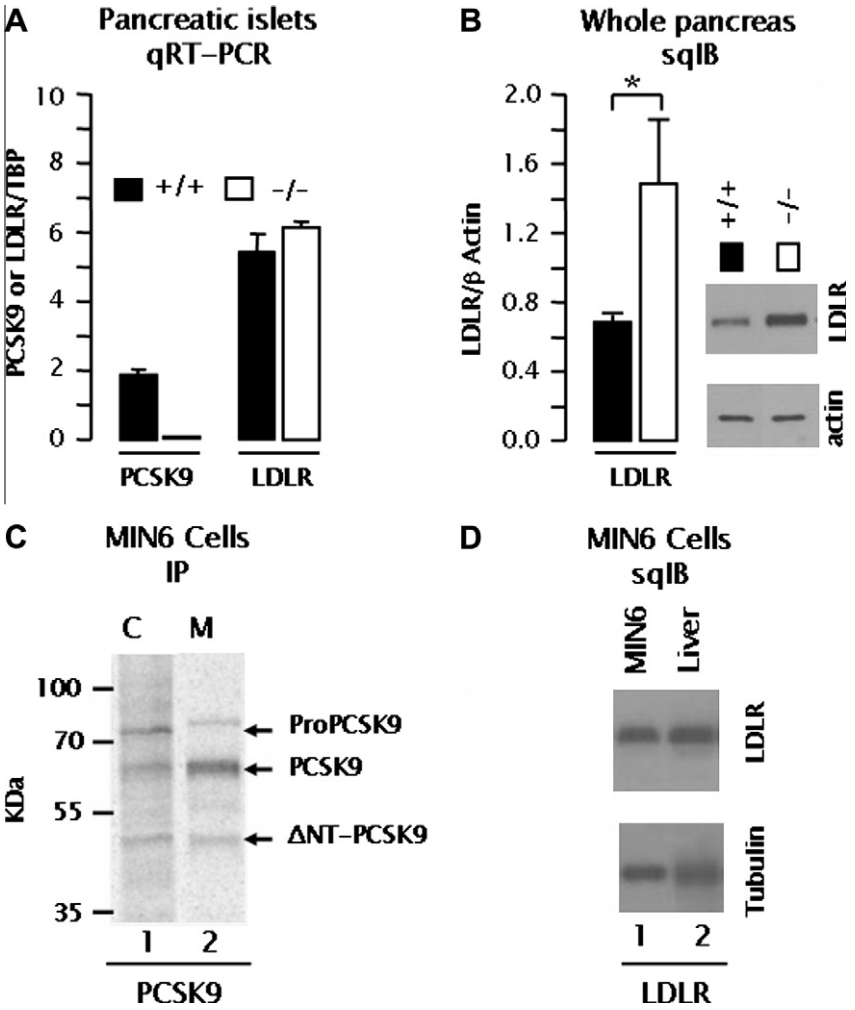

Fig. 1. Mouse pancreatic β cells express PCSK9 and LDLR. *Pcsk9*$^{+/+}$ or *Pcsk9*$^{-/-}$ male mice were used (n = 4/genotype). (A) Isolated pancreatic islets were used as the source of RNA for qRT-PCR for PCSK9 and LDLR and TBP mRNAs, the latter serving as normalizing internal control. (B) Whole pancreas extracts (n = 4 mice/genotype) were analyzed by sqIB for LDLR (120 kDa) and β actin (35 kDa), the latter serving as a normalizing internal control. *Pcsk9*$^{-/-}$ mice contained twofold ($P < 0.05$) more LRLR protein in their pancreas than the *Pcsk9*$^{+/+}$ mice. (C) MIN6 cells were metabolically labeled with [^{35}S]-Met/Cys for 6 h. The cell lysate and spent medium were analyzed by IP of PCSK9-related forms. (D) MIN6 cell lysates and liver extracts from *Pcsk9*$^{+/+}$ or *Pcsk9*$^{-/-}$ mice were analyzed by sqIB for LDLR (120 kDa) and α-tubulin (55 kDa). Note the increase in the receptor level in hepatic extracts from null mice.

CA). Standard curves were established using varying amounts of pre-quantified amplicons of each transcript. The level of mRNA for the TATA-box binding protein (TBP) was used for normalization.

2.6. Histology

Paraffin sections (5 μm thickness) were deparaffinized using CitriSolv and rehydrated with graded ethanol (100%, 95%, and 75% ethanol) and H_2O. Gill's hematoxylin solution (No. 2) was used for nuclear staining and 0.5% eosin solution for cytoplasm staining. Apoptosis, tubular complexes and inflammation were evaluated morphologically using previously defined characteristics [19].

2.7. Metabolic labeling, immunoprecipitation (IP) and immunoblotting (IB)

The details of these procedures are described in Supplementary data.

2.8. Statistical analyses

Results are expressed as means ± standard errors of means (SEM). Differences between experimental groups were analyzed by unpaired Student *t*-test. Significance of differences was set at $P < 0.05$.

3. Results

3.1. PCSK9 and LDLR expression in pancreatic islets

PCSK9 mRNA was below detection by qRT-PCR in RNA extracted from whole pancreas [3]. Because pancreatic islet cell lines have been shown to contain this transcript [2], we isolated pancreatic islets from *Pcsk9*$^{+/+}$ and *Pcsk9*$^{-/-}$ mice and analyzed their RNA by qRT-PCR for both PCSK9 and LDLR mRNA levels using TBP mRNA level for normalization. LDLR transcripts were present at similar levels in islets from *Pcsk9*$^{+/+}$ and *Pcsk9*$^{-/-}$ mice; *Pcsk9* transcripts were present in islets from *Pcsk9*$^{+/+}$ mice only (Fig. 1A).

Semi-quantitative immunoblotting (sqIB) analysis of proteins extracted from whole pancreas showed that *Pcsk9*$^{-/-}$ mice contained twofold more LDLR in this organ than *Pcsk9*$^{+/+}$ mice ($P < 0.05$) (Fig. 1B). Since the anti-mouse PCSK9 antibody available to us unambiguously recognized this protein in mouse plasma but not tissue extracts and only by IP [3], we examined for possible co-expression of PCSK9 and LDLR in mouse insulinoma MIN6 cells. These cells were grown in medium containing ^{35}S-Met/Cys to met-

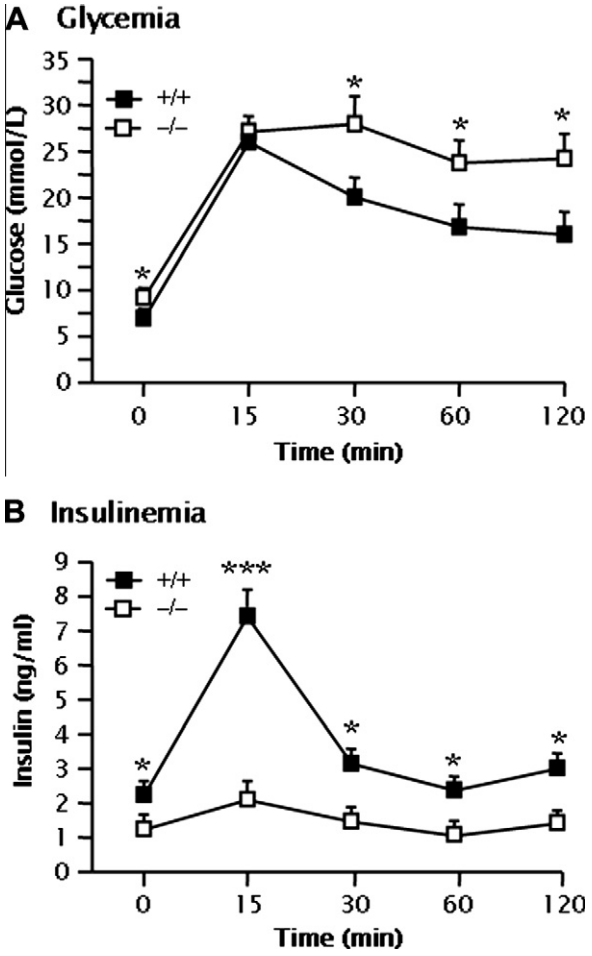

Fig. 3. OGTT. *Pcsk9*$^{+/+}$ and *Pcsk9*$^{-/-}$ male mice ($n = 9$/genotype) were subjected to an OGTT. (A) Plasma glucose was not different between the two genotypes at 15 min, but was significantly higher in mutant mice at later time points. (B) Plasma insulin was significantly lower in mutant mice at all time points, most noticeably at 15 min. *$P < 0.05$; ***$P < 0.001$.

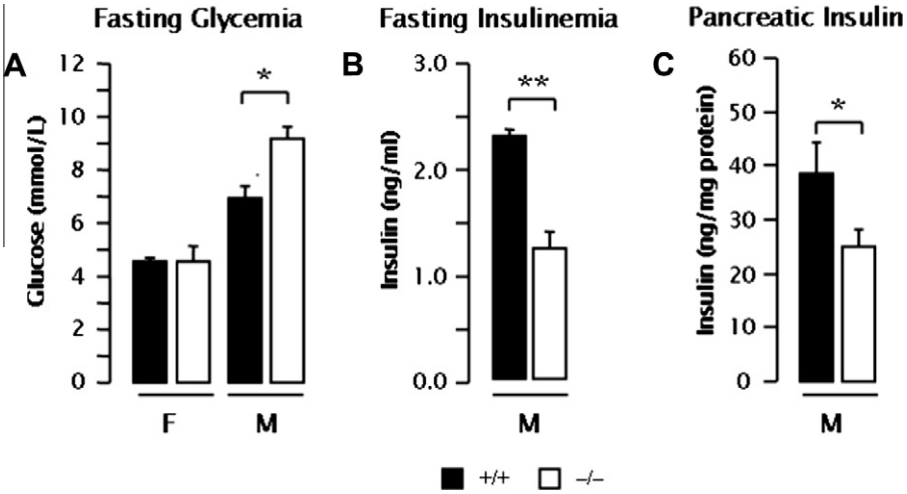

Fig. 2. Glucose homeostasis is altered in PCSK9-deficient mice. Female (F) and male (M), *Pcsk9*$^{+/+}$ and *Pcsk9*$^{-/-}$ 4-month-old mice ($n = 8$/gender/genotype) were phenotyped for glucose homeostasis parameters. (A) Fasting glycemia was similar in females; but was increased ($P < 0.05$) in male *Pcsk9*$^{-/-}$ mice. (B) Insulinemia was reduced ($P < 0.01$) in male *Pcsk9*$^{-/-}$ mice. (C) Pancreatic insulin content was reduced ($P < 0.05$) in *Pcsk9*$^{-/-}$ mice.

abolically label *de novo* biosynthesized proteins. Spent media and cell lysates were subjected to IP using the anti-mouse PCSK9 antibody and the precipitates analyzed by SDS–PAGE followed by phosphorimaging. Cell lysates contained immunoreactive proteins of 72, 62, and 50 kDa corresponding to the mass expected for proPCSK9, PCSK9, and the furin-cleaved ΔNT_{218}-PCSK9, respectively (Fig. 1C, lane 1) (see also diagrams in Supplementary Fig. 1S). Spent media contained predominantly the 62-kDa form (Fig. 1C, lane 2). By IB, LDLR was detected in MIN6 cells at levels comparable to that of normal liver (Fig. 1D, lane 1 *vs* 2).

3.2. Plasma glucose, plasma insulin and glucose tolerance

Before 4 months of age, there was no significant difference in fasting plasma glucose and insulin between *Pcsk9*$^{-/-}$ and *Pcsk9*$^{+/+}$ mice within gender. At 4 months, fasting plasma glucose was significantly higher in male *Pcsk9*$^{-/-}$ mice relative to *Pcsk9*$^{+/+}$ mice ($P < 0.05$), but remained similar between genotypes in female mice (Fig. 2A). Subsequent studies were conducted with male mice only. Fasting plasma insulin in *Pcsk9*$^{-/-}$ mice was about half that of *Pcsk9*$^{+/+}$ mice ($P < 0.01$) (Fig. 2B). The pancreas from *Pcsk9*$^{-/-}$ mice contained ~30% less insulin than those from *Pcsk9*$^{+/+}$ mice ($P < 0.05$) (Fig. 2C), suggesting that the mutant may suffer from insulin insufficiency.

In an OGTT, glycemia was elevated but similar between the two genotypes at 15 min post-oral glucose administration; at subsequent time points up to 120 min, it declined in *Pcsk9*$^{+/+}$ mice but remained significantly higher in *Pcsk9*$^{-/-}$ mice ($P < 0.05$) (Fig. 3A). At 15 min, insulinemia dramatically increased in *Pcsk9*$^{+/+}$ mice, but barely changed in *Pcsk9*$^{-/-}$ mice ($P < 0.001$); it returned to pre-gavage levels at subsequent time points (Fig. 3B).

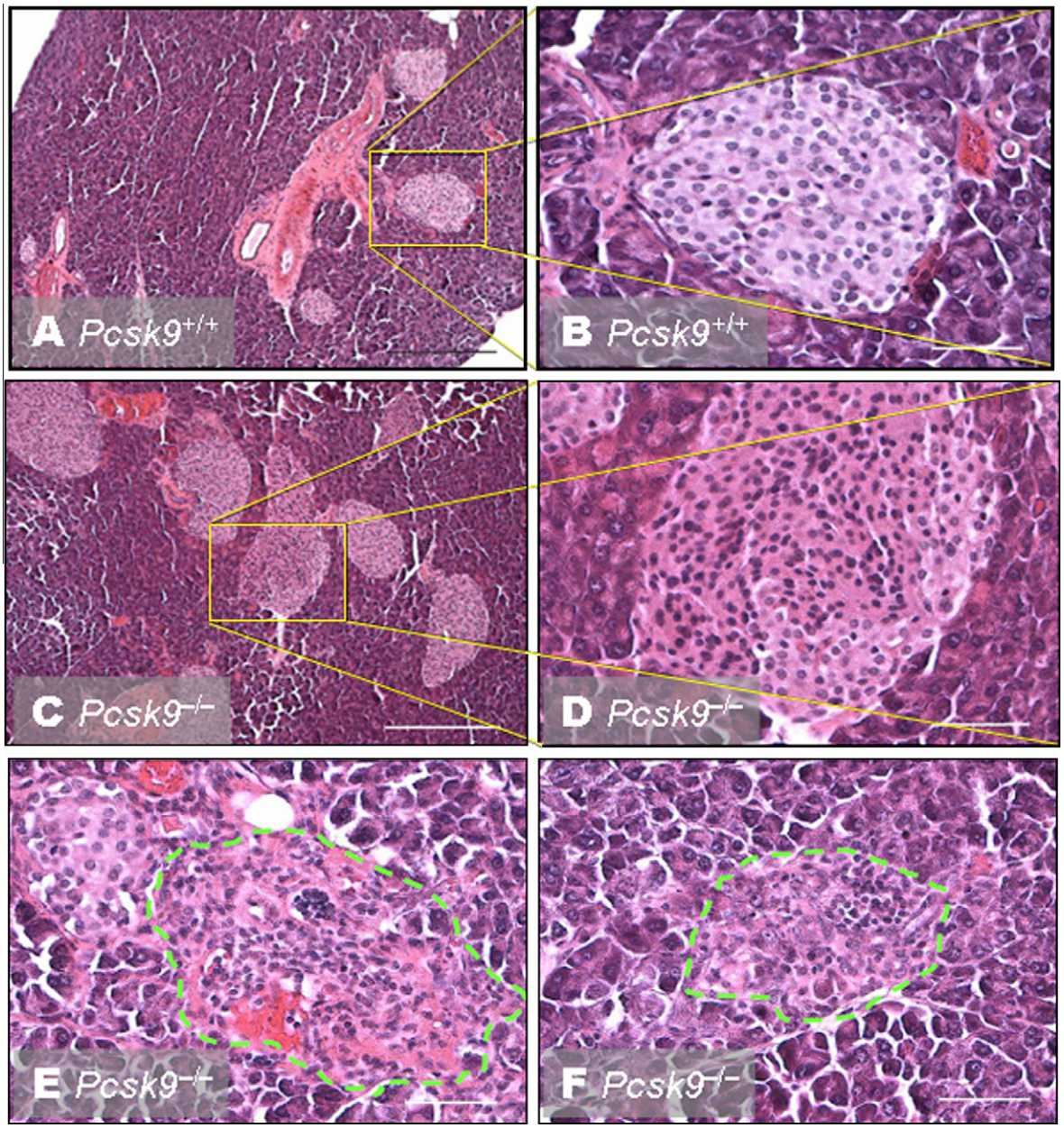

Fig. 4. Pancreatic histology. Whereas pancreatic islets from *Pcsk9*$^{+/+}$ mice appeared generally normal (panels A and B), those for *Pcsk9*$^{-/-}$ often exhibited irregular shapes (panel C), with most β-cells displaying eosinophilic cytoplasm and condensed nuclei (panel D), suggesting early apoptosis. Also observed in the pancreas of these mutant mice were sites of inflammation (outlined in panel E) and occasional tubular complexes (outlined in panel F). Bar = 250 μm in panels (A) and (C); 50 μm in panels (B), (D–F).

3.3. Islet histology

Histological analysis of pancreas sections indicated that, compared to *Pcsk9*$^{+/+}$ mice (Fig. 4A and B), *Pcsk9* $^{-/-}$ mice often exhibited islets of irregular shapes (Fig. 4C), with most β-cells displaying eosinophilic cytoplasm and condensed nuclei (Fig. 4D), suggestive of early apoptosis. Also observed in the pancreas of these mutant mice were sites of inflammation (Fig. 4E) and occasional tubular complexes (Fig. 4F). These complexes represent transitional structures involved in islet neogenesis [19].

4. Discussion

Data presented in this report show that, as they age, PCSK9-deficient male mice exhibit mild chronic hyperglycemia and impaired glucose tolerance. Their pancreatic content and plasma levels of insulin are reduced, suggesting insulin insufficiency. Their pancreatic islets are morphologically abnormal, showing signs of inflammation and early apoptosis. Cholesterol accumulation in the islets of PCSK9-deficient could be a cause of these abnormalities, considering mounting experimental evidence of the toxic effect of accumulation of this sterol in β cells [20,21]. This accumulation is an expected consequence of the lack of PCSK9-mediated down regulation of pancreatic islet LDLR and of LDL-C uptake *via* this receptor.

However, an LDLR-independent effect of PCSK9 deficiency remains a possibility. Experimental evidence suggests that this protein may play a role in organ development and repair. Elevated expression of its mRNA precedes liver regeneration following partial hepatectomy [3]. Furthermore, liver from PCSK9-null mice cannot regenerate following partial hepatectomy and undergoes necrosis [3]. Pancreatic islets can be renewed and the pancreas can regenerate following partial pancreatectomy [22]. It would be interesting to determine whether PCSK9 plays any role in this process.

Gender, age, modifier genes, and diet are factors known to contribute to deterioration of glucose homeostasis in susceptible subjects. In PCSK9-null male mice, this deterioration becomes observable at 4 months of age. The reduced susceptibility of female mice to this process has been observed with other mouse models [23,24]. This dichotomy is attributed to the beneficial effects of female sex hormones on β cell functions [25].

The relevance of this mouse model of global PCSK9 deficiency to the pathogenesis of diabetes in humans is unclear. Although several *PCSK9* single-nucleotide polymorphisms associated with persistently low plasma cholesterol have been identified in the human genome [26], no case of *PCSK9* nullizygosity has ever been reported. Interestingly, in a 1984 large California study comparing individuals across deciles of plasma cholesterol levels for sociologic, biologic, and medical characteristics, a greater prevalence of diabetes was unexpectedly noted in individuals in the lowest decile of cholesterolemia [27]. These diabetics also exhibited a greater body mass index and higher plasma triglycerides levels than non-diabetics in the same decile or diabetics in higher deciles, suggesting that other metabolic dysfunctions contributed to this association. Whether PCSK9 deficiency could influence the onset of such a syndrome will require more studies.

In conclusion, data presented in this report suggest total PCSK9 deficiency may deleteriously affect glucose homeostasis.

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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.febslet.2009.12.018.

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