PCSK9 deficiency unmasks a sex- and tissue-specific subcellular distribution of the LDL and VLDL receptors in mice

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Abstract  Proprotein convertase subtilisin kexin type 9 (PCSK9), the last member of the family of Proprotein Convertases related to Subtilisin and Kexin, regulates LDL-cholesterol by promoting the endosomal/lysosomal degradation of the LDL receptor (LDLR). Herein, we show that the LDLR cell surface levels dramatically increase in the liver and pancreatic islets of PCSK9 KO male but not female mice. In contrast, in KO female mice, the LDLR is more abundant at the cell surface enterocytes, as is the VLDL receptor (VLDLR) at the cell surface of adipocytes. Ovariectomy of KO female mice led to a typical KO male pattern, whereas 17β-estradiol (E2) treatment restored the female pattern without concomitant changes in LDLR adaptor protein 1 (also known as ARH), disabled-2, or inducible degrader of the LDLR expression levels. We also show that this E2-mediated regulation, which is observed only in the absence of PCSK9, is abolished upon feeding the mice a high-cholesterol diet. The latter dramatically represses PCSK9 expression and leads to high surface levels of the LDLR in the hepatocytes of all sexes and genotypes. In conclusion, the absence of PCSK9 results in a sex- and tissue-specific subcellular distribution of the LDLR and VLDLR, which is determined by E2 levels.—Roubtsova, A., A. Chamberland, J. Marcinkiewicz, R. Essalmani, A. Fazel, J. J. Bergeron, N. G. Seidah, and A. Prat. PCSK9 deficiency unmasks a sex- and tissue-specific subcellular distribution of the LDL and VLDL receptors in mice. J. Lipid Res. 2015. 56: 2133–2142.

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PCSK9 belongs to a family of serine proteases called the proprotein convertases (PCs) because they cleave and often activate precursors of growth factors, hormones, neuropeptides, receptors, and surface glycoproteins (1).

These PCs are encoded by genes designated PCSK1 to PCSK9, except for the third and eighth members furin and SKI-1/S1P, whose genes were named FURIN and MBTPSI, respectively. PCSK9 was the third gene known to be involved in autosomal dominant hypercholesterolemia (2) after those encoding the LDL receptor (LDLR) and apolipoprotein B. PCSK9 binds to the LDLR and directs it to the endosomal/lysosomal pathway for degradation (3, 4). Gain-of-function mutations in PCSK9 were shown to enhance the degradation of the LDLR (2, 4), with ensuing increased LDL-cholesterol levels in plasma. Conversely, PCSK9 loss-of-function mutations result in hypocholesterolemia (5). Low concentrations of active PCSK9 are also associated with a lower incidence of atherosclerosis (6), myocardial infarction (7), and stroke (8). Individuals entirely lacking functional PCSK9 are healthy and have extremely low levels of LDL-cholesterol (∼0.4 mM) (9–11). Accordingly, monoclonal antibodies directed against PCSK9 that disrupt the PCSK9-LDLR interaction are efficient in reducing LDL-cholesterol levels (by >50%) and are commercially available (reviewed in Ref. 12).

Like all other PCs, PCSK9 is synthesized as a precursor (proPCSK9; 74 kDa) that undergoes an autocatalytic cleavage at the VFAQ152 site, allowing it to exit the endoplasmic reticulum (4, 13). However, the inhibitory prosegment remains tightly bound to PCSK9 (14, 15), and the complex is secreted. Thus, mature PCSK9 has no catalytic activity in trans but acts as a binding protein to specific receptors.

Abbreviations:  Dab2, disabled-2; E2, 17β-estradiol; IDOL, inducible degrader of the LDL receptor; LDLR, low-density lipoprotein receptor; LDLRAP1, LDLR adaptor protein 1; OxE2, ovariectomy followed by E2 treatment; OxP, ovariectomy followed by placebo treatment; PC, proprotein convertase; PCSK9, proprotein convertase subtilisin kexin type 9; PM, plasma membrane; VLDLR, very low density lipoprotein receptor.

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The catalytic domain of PCSK9 binds to the EGF-A domain of the LDLR, and the resulting PCSK9-LDLR complex is then directed to endosomes/lysosomes for degradation by a still undefined pathway (1, 16, 17). The most powerful gain-of-function mutation in PCSK9, D374Y (18), enhances by more than 10-fold the affinity of PCSK9 for the LDLR (15).

In mice, PCSK9 is highly expressed in liver, pancreatic islets, intestine, and kidney (14, 19–21). However, circulating PCSK9 originates exclusively from hepatocytes (19, 22) and can regulate peripheral receptors in tissues that do not express PCSK9 (e.g., VLDLR in adipose tissue) (22). In a previous study, we showed that the absence of circulating PCSK9 led to increased levels of surface VLDLR in the perigonadal tissue. Intriguingly, the increase was almost 10-fold higher in female mice (22). Because no data were available on the LDLR distribution in female mice, which are often not analyzed, we compared the surface levels of the LDLR in the liver of PCSK9 KO mice of both sexes. Although PCSK9 KO female mice express higher surface levels of the VLDLR in perigonadal adipose tissue than male mice, they express lower levels of the LDLR at the hepatocyte cell surface. This was confirmed by the analysis of liver plasma membrane (PM) fractions. This phenotype is dependent on female hormones: ovariectomy generated a male pattern (i.e., low surface VLDLR levels in hepatocytes) that was reversed to a female pattern upon treatment of ovariectomized mice with 17β-estradiol. In contrast, feeding KO mice with a high-cholesterol diet led to high surface levels of the LDLR in both sexes.

MATERIALS AND METHODS

Animals, treatments, and plasma analyses

All procedures were approved by the IRCM animal care committee. WT (wild-type), LDLR (23), and PCSK9 KO (19) mice were on the C57BL/6 background, housed under 12-h light/dark cycles, and fed chow (2018 Teklad Global) or a high-cholesterol (0.425%) diet (TD.08464 Teklad, Harlan Laboratories, Madison, WI). Mice were bled and euthanized at 3 months of age after fasting for 3 h. Blood was collected in heparin-coated Microtainer tubes (BD Biosciences, Fisher, ON), centrifuged for 15 min at 850 g at 4°C, and kept at −80°C until assayed. For plasma17β-estradiol (E2) measurement, mice were bled at the day of proestrus, as estimated from smears of vaginal cells, using the Estradiol EAI Kit (Cayman Chemical, Ann Arbor, MI). Plasmatic PCSK9 and LDL concentration were measured using the CircuLex mouse/rat PCSK9 (MBL, Woburn, MA) and Low Density Lipoprotein (Cloud-Clone Corp., Houston, TX) ELISA kits, respectively.

Immunohistochemistry

For LDLR staining, mice were euthanized, and one lobe of the liver was frozen in ice-cold isopentane and kept at −80°C. Cryosections (6 μm thick) were immediately fixed in 4% paraformaldehyde in PBS for 1 h, rinsed three times in PBS, and blocked in 2% BSA (Sigma-Aldrich, Oakville, ON) in PBS for 1 h at room temperature. Sections were then incubated overnight at 4°C with a goat antibody against mouse LDLR (1:150; R&D systems, Minneapolis, MA) in PBS containing 1% BSA and washed three times for 10 min in PBS. For VLDLR visualization, thick paraffin sections (8 μm) of perigonadal adipose tissue were unparaffinized, blocked, and incubated as above, using a goat antibody against mouse VLDLR (1:150; Santa Cruz Biotechnology, Santa Cruz, California). Labeling was visualized by incubation with Alexa Fluor 488-labeled anti-goat IgGs (1:150; Invitrogen, Burlington, ON) for 1 h at room temperature in PBS. After three 10 min washes, nuclei were counterstained with Hoechst dye (Sigma). After two short washes, the sections were dipped in 70% ethanol and closed using 90% glycerol. Images were acquired as described previously (22). Five to nine mice were analyzed by sex and genotype. In all cases, the most representative image was chosen.

Ovariectomy and E2 replacement

Three-month-old female mice under 2% isoflurane (CDMV, Quebec, QC) in oxygen at a flow rate of 1 L/min underwent sham surgery (sham) or ovariectomy followed by placebo (OvxP) or E2 (OvxE2) treatment. A 1 cm skin incision over the spinal cord was made, and ovaries were removed by sectioning the fallopian tubes. Skin was closed with 5-0 PDS®II violet monofilament sterile suture (Ethicon, Somerville, NJ). Two days later, mice were anesthetized as above, and pellets containing placebo or 25 μg of E2 (Innovative Research of America, Sarasota, FL) were introduced subcutaneously between the ear and shoulder. After 21 days of placebo or E2 release, mice were bled and euthanized.

Quantitative RT-PCR

Total RNA from liver was extracted with TRIzol (Invitrogen). cDNA was generated from 250 ng of total RNA using a SuperScript II cDNA reverse transcriptase (Invitrogen). Quantitative PCR was done using the SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA) and the following sense and antisense primers: PCSK9, 5′-CAGGAGACACATTGCATCC and 5′-TGCAAATCAGGAGCATGGG; LDLR, 5′-GGAGATGCACTTGCCATCCT and 5′-AGGCTGTCCCCCCCAAGAC; and HMG-CoA reductase, 5′-GAAGAGCAGCAACATGCTGAAA and 5′-GACTGCGAGAATCTGCAATGT. Each gene expression was normalized to that of hypoxanthine phosphoribosyltransferase (5′-CAGGAGATTTGGAAAAATGTGTT and 5′-CTTCCATGACATCCTGGAAGA) or TATA box binding protein (5′-GCTGAATATAATCCCAAGCGATT and 5′-CAGTGTCCCCGCTCCT).

Protein analyses

Protein extracts (30 μg/well if not indicated) were separated on 8% Tris-glycine gels and transferred to 0.45 μm PVDF membranes (PerkinElmer, Woodbridge, ON). Membranes were blocked for 1 h at room temperature in TBS containing 0.1% (v/v) Tween 20 and 5% (w/v) dry milk and incubated overnight at 4°C in the same buffer with mouse LDLR (1:1,000; see above), STIP1 (1:1,000; Abcam, Cambridge, MA), and β-actin (1:5,000; Sigma) antibodies. Staining was revealed with corresponding HRP-labeled secondary antibodies and ECL Western blotting substrate (Pierce, Rockford, IL). Signals on films were quantified using the ImageJ 1.29v software. Signals of liver homogenates and plasma membrane fractions were obtained with the Clarity Western ECL Substrate (Bio-Rad Laboratories, Mississauga, ON) and the more sensitive ChemiDoc MP System (Bio-Rad Laboratories) and were quantified by ImageLab 5.2 software. All values were normalized to that of β-actin and calibrated by setting the control ratio to 1.

PM purification, measurement of the 5′ nucleotidase activity, and electron microscopy analysis

In each of three experiments, two livers of WT and KO male and female mice (n = 8 mice) were homogenized in 0.25 M sucrose
containing 5 mM Tris-HCl (pH 7.4), 0.5 mM MgCl₂, and protease inhibitors (Complete EDTA-free; Roche Diagnostics, Mannheim, Germany). PM preparation was performed as previously described (24) with a few modifications (25). Briefly, the homogenates were filtered on gauze, and aliquots were kept for analysis. After two centrifugations at 400 g to remove nuclei and tissue debris and a third one at 1,500 g to collect membranes, the latter were resuspended in 1.8 M buffered sucrose and loaded under two layers of 1 M and 1.42 M buffered sucrose. After ultracentrifugation, the 1 M-1.42 M interface was collected, centrifuged, resuspended in 1.8 M buffered sucrose, and reloaded at the bottom of a second gradient, except for the first experiment. Membranes were collected at the 0.25 M-1.42 M interface, centrifuged, and resuspended in buffered 0.25 M sucrose (PM fractions). The 5′ nucleotidase activity was then evaluated in homogenates and PM fractions (26). An aliquot of each preparation was incubated for 15 min at 37°C in a buffer containing 80 mM Tris-HCl (pH 8.5), 15 mM MgCl₂, 0.15% Triton-X100, and 15 mM 5′-AMP. The reaction was stopped with trichloroacetic acid (12.5% final) and placed on ice for 20 min. After centrifugation, the supernatant was analyzed for its phosphorus content using the method of Ames and Dubin (27). Finally, a fresh aliquot of the PM fractions was fixed for EM analysis as described previously (28).

Statistical analysis

Data were analyzed using the Excel software for SEM and two-tailed Student’s t test calculations.

RESULTS

LDLR subcellular distribution is affected by sex in PCSK9 KO mice

We have reported previously that, in perigonadal adipose tissue, surface VLDLR levels were increased in PCSK9 KO mice versus WT mice, by 4-fold in male mice and by ~40-fold in female mice (22). To assess whether surface LDLR levels in the liver were also affected by sex in PCSK9 KO mice, we first compared LDLR protein levels in liver extracts (Fig. 1A). As reported previously for PCSK9 KO male mice (19, 29), LDLR levels were ~2-fold higher. A similar increase was observed in extracts from female mice, indicating that there were no sex differences in the total amount of LDLR in the liver. Furthermore, LDLR mRNA levels were neither affected by genotype nor sex (Fig. 1B). LDLR immunostaining of liver cryosections, however, revealed that the surface fraction of LDLR was dramatically increased in KO male mice but was not affected in KO female mice (Fig. 1C). As a negative control, the LDLR staining was simultaneously analyzed in liver cryosections from LDLR-deficient mice (not shown) and found to give background values. Thus, in the absence of PCSK9, surface LDLR levels were higher in male livers than in female ones. The quasiasence of LDLR labeling in WT sections is due to the massive accumulation of the cell surface signal in KO male mice because the exposure time was set on the latter sections to avoid any saturation and was kept unchanged throughout the acquisition of the pictures.

PCSK9 and LDLR are also highly expressed in pancreatic islets (20, 21) and small intestine (14). As in the liver, total LDLR protein levels were ~2-fold higher in PCSK9 KO mice as compared with WT mice in pancreas and 1.3-fold higher in duodenum (Fig. 2). In addition, LDLR mRNA levels were unchanged in KO male and female mice in duodenum (Fig. 2B), in agreement with the role of PCSK9 in the post-translational regulation of the LDLR. Like in the liver, LDLR labeling was strongest in male KO pancreatic islets than in female ones. However, different from the liver and pancreas, LDLR labeling in duodenum was strongest in KO female mice (Fig. 2B). This suggests that the absence of PCSK9 favors LDLR accumulation at the cell surface in a sex- and tissue-dependent manner.

In vivo, the fraction of the visualized LDLR at the cell surface of hepatocytes or enterocytes likely represents a minor proportion of the total amount of LDLR protein because the latter does not seem to differ between PCSK9 KO male and female mice (Figs. 1, 2).

The absence of PCSK9 leads to increased LDLR protein levels in male, but not female, PM fractions

Evidence of an accumulation in the liver of the LDLR at the cell surface of male, but not female, hepatocytes in the absence of PCSK9 was also obtained by Western blot analysis of PM preparations. In each of three experiments, two livers per genotype/sex were homogenized. PM fractions were obtained by successive sucrose gradients and contained

Fig. 1. Surface LDLR levels are higher in male than in female PCSK9 KO livers. A: LDLR protein levels were analyzed by Western blotting in the liver of WT (open bars) or PCSK9 KO (gray bars) male and female mice. LDLR band intensities were quantified by densitometry and normalized to that of β-actin. *P < 0.05. WT male
0.5% to 1% of the original protein content (~2 mg out of ~320 mg). Their quality was supported by an ~10- to 18-fold enrichment in 5′ nucleotidase activity (Table 1) and the predominance of large sheets of plasma membranes without major contamination as seen upon electron microscopy analysis (supplementary Fig. 1).

To quantify the LDLR signal, 30 μg and 6 μg of proteins from homogenates and PM fractions, respectively, were loaded on the same gel (Fig. 3A). The LDLR signal by itself exhibited a variable enrichment factor from ~20 to ~45, possibly reflecting slightly different metabolic states. The cytosolic protein STIP1 was partially lost in PM fractions (enrichment factor of 0.32, taking into account the 5 times lower loading for PM fractions). In contrast, β-actin, which is known to associate with cell membranes, presented a similar enrichment (11- to 18-fold) to that of 5′ nucleotidase activity. Because β-actin was very stable and correctly quantified in both homogenates and PM fractions, it was chosen together with the 5′ nucleotidase activity to normalize the LDLR signal (Fig. 3B). KO male and female homogenates exhibited, respectively, 3.0- and 4.6-fold higher LDLR signals than the WT homogenates. These ratios, which are higher than those presented in Fig. 1, reflect the greater sensitivity of the Bio-Rad ChemiDoc MP System used to quantify chemiluminescent signals in these experiments.

The LDLR signal in PM fractions was 3.4-fold higher in KO male mice than in WT male mice, revealing a similar increase in KO homogenates and PM fractions. In contrast, in female mice, the 4.6-fold higher level of LDLR signal observed in KO homogenates did not lead to a higher proportion of the LDLR in the PM fractions (Fig. 3B).

**Sexual dimorphism in LDLR distribution in KO livers is mainly mediated by E2**

To assess whether the amount of cell surface LDLR was sensitive to female hormones, sham surgeries or ovariectomies

| Mice         | Homogenates (n = 3) | PM fractions (n = 3) | Enrichment Factors |
|--------------|---------------------|----------------------|--------------------|
|              | Exp. 1  | Exp. 2  | Exp. 3  | Exp. 1  | Exp. 2  | Exp. 3  | Exp. 1  | Exp. 2  | Exp. 3  |
| WT male      | 15.5   | 19.8   | 9.1    | 109    | 252    | 129    | 7.1    | 12.7   | 14.2   |
| KO male      | 13.0   | 18.5   | 7.8    | 116    | 303    | 138    | 8.9    | 16.4   | 17.6   |
| WT female    | 15.9   | 23.0   | 10.8   | 182    | 312    | 266    | 11.5   | 13.6   | 24.5   |
| KO female    | 11.8   | 20.5   | 14.0   | 178    | 320    | 207    | 15.1   | 15.6   | 14.7   |
| Average      | 14.1   | 20.5   | 10.5   | 146    | 297    | 185    | 10.4   | 14.6   | 17.8   |

The average enrichment factors in experiments 1, 2, and 3 are shown in bold.
E2 treatment does not affect PCSK9 mRNA levels but enhances its circulating levels

Female mice exhibited 1.5-fold higher circulating PCSK9 levels than male mice (P < 0.0005) (Fig. 5B) and ~50% higher PCSK9 mRNA levels, suggesting that higher circulating levels of the protein reflect the higher expression of the gene in female mice. Although not affected by ovariectomy, circulating PCSK9 levels were ~2-fold increased by E2 treatment (Fig. 5B). Does E2 up-regulate PCSK9 gene expression? In WT ovariectomized mice, PCSK9 and HMG-CoA reductase mRNA levels decreased by 0.7- and 0.6-fold, respectively, but returned to sham levels after E2 treatment (Fig. 5C), suggesting their mRNA levels are only sensitive to physiological levels of E2. LDLR mRNA levels are more stable because they were not affected by ovariectomy and increased by 30% upon E2 treatment. In PCSK9 KO mice, LDLR and HMG-CoA reductase mRNA levels seem less sensitive to E2 variations. Thus, in C57BL/6 mice, the mRNA levels of three genes known to be regulated by SREBP-2 and to belong to the cholesterol synthesis pathway were slightly reduced in the absence of E2 and unaffected by a 15-fold excess of E2, as observed previously in rats by Ngo et al. (30). Thus, because PCSK9 mRNA levels were not higher in E2-treated mice as compared with sham mice, the ~2-fold increase in

were performed on mice of 10 to 12 weeks of age (n = 8–9). After 48 h, ovariectomized female mice received subcutaneous pellets containing either placebo or 25 μg of E2. After 21 days, mice were euthanized and analyzed. WT and KO uteri were undistinguishable (not shown). The shrinking of the uterus observed in ovariectomized females treated with placebo (OvxP) was largely prevented in female mice supplemented with E2 (OvxE2) (Fig. 4A), revealing the efficacy of the hormonal replacement. In KO mice, ovariectomy plus placebo treatment did not affect total LDLR protein levels in the liver (Fig. 4B) but led to a strong increase of the cell surface immunohistochemical LDLR labeling (Fig. 4C), comparable to that seen in KO male mice. This increase was completely reversed by E2 treatment (OvxE2). The latter treatment even seems to further reduce the surface LDLR signal as compared with that of sham mice (data not shown), possibly due to the 15-fold higher than normal concentrations of E2 achieved by the E2-releasing pellets (Fig. 5A).
The lack of PCSK9 was shown to favor perigonadal and perirenal fat accumulation and adipocyte hypertrophy (22), suggesting that PCSK9, like estrogens, prevents visceral fat accumulation (36). We have thus analyzed their combined effects on the ratio of perigonadal adipose tissue weight to body weight in each group (Fig. 6C). Ovariectomy alone had no impact on this ratio, but the lack of PCSK9 led to increases of perigonadal fat mass by 27% in sham mice and 74% in ovariectomized mice. Notably, the combination of the lack of PCSK9 and ovaries resulted in a 2.2-fold increase in fat mass. In addition, E2 supplementation (OvxE2 vs. sham) was less efficient in reducing the perigonadal fat mass in KO mice (73%) than in WT mice (73%). As a consequence, OvxE2 KO mice exhibited 156% more perigonadal fat mass than OvxE2 WT mice (Fig. 6C).

**Sex-dependent VLDLR subcellular distribution in PCSK9 KO adipose tissue is mainly mediated by E2**

In the perigonadal adipose tissue of PCSK9 KO mice, the accumulation of surface VLDLR is stronger in female than in male mice (22). To verify whether this difference was also dependent on female hormones, the adipose tissues from the above sham or ovariectomized mice were examined (Fig. 6A). The strong surface VLDLR labeling observed in sham PCSK9 KO mice almost disappeared upon ovariectomy (OvxP) and was restored by E2 treatment (OvxE2). Thus, in the absence of PCSK9, E2 seems to favor VLDLR accumulation at the cell surface of female perigonadal adipocytes but seems to reduce LDLR accumulation at the cell surface of their hepatocytes.

**Synergistic effect of ovariectomy and PCSK9 deficiency on perigonadal fat mass**

The body weight of KO mice versus that of WT mice was found to be slightly increased (10–16%) (Fig. 6B) as reported previously (22). Ovariectomy increased WT and KO body weights by 7% and 12%, respectively, whereas E2 treatment restored them to their original value.

**PCSK9-independent LDLR regulation**

Among the factors known to regulate LDLR endocytosis, and thus surface LDLR levels, are the adaptor proteins

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**Fig. 5.** E2 treatment does not affect PCSK9 mRNA levels but enhances its circulating levels. Plasma E2 (A) and PCSK9 (B) levels after 3 h of fasting [n = 9–17 for untreated male (M) or female (F) mice; n = 8–9 for sham, OvxP, or OvxE2 mice]. C: Liver PCSK9, LDLR, and HMG-CoA reductase mRNA levels (n = 7–9). Values are average ± SEM. *P < 0.05; **P < 0.005; ***P < 0.0005.

**Fig. 6.** PCSK9 and estrogens regulate the VLDLR subcellular distribution and fat accumulation in perigonadal depots. A: VLDLR immunohistochemistry on paraffin sections was performed on 7 to 9 mice per sex and genotype. A representative picture is shown. Body weights (B) and percentages of perigonadal fat (C) are shown. Each bar represents the average ± SEM (n = 8–9). *P < 0.05; **P < 0.005; ***P < 0.0005.
able to show that this process was lost when mice of 13 weeks of age were fed for 2 weeks a chow diet containing 0.42% cholesterol versus 0%. To our surprise, although chow-fed control mice exhibited the classical LDLR pattern, high dietary cholesterol levels resulted in a dramatic increase of the LDLR labeling in all genotypes and sexes (Fig. 7A): the sex difference was lost in KO mice, with female mice presenting a strong LDLR labeling similar to male mice and with WT mice exhibiting a pattern similar to that of KO mice.

In agreement, a quasi-loss of circulating PCSK9 (Fig. 7B) and 3- to 4-fold lower PCSK9 mRNAs (Fig. 7C) indicate that WT mice are now metabolically closer to KO mice. However, the more dramatic reduction in circulating PCSK9 than expected from its mRNA levels indicates an increased clearance of PCSK9. Higher surface LDLR levels may favor the uptake of the remaining PCSK9, and thus LDLR degradation, leading to a new equilibrium characterized by more LDLRs at the cell surface of hepatocytes. LDLR mRNA levels were unaffected, and this may explain why total LDLR protein levels did not increase (Fig. 7D) but remained stable in WT livers. A similar observation was done with Pcsk9<sup>+/−</sup> (heterozygote) mice that presented a 70% drop in circulating PCSK9 levels, likely due to a concomitant increase in LDLR levels (42). Whether this unexpected accumulation of surface LDLR in WT mice fed a high-cholesterol diet contributes to the protection of vessel walls by clearing the excess of circulating cholesterol remains to be verified.

In PCSK9-deficient mice, LDLR protein levels dropped to those of WT mice concomitantly with a 40% drop in their mRNA levels. This latter drop suggests that KO male and female mice had an increased LDLR activity that led to a stronger down-regulation of the cholesterogenic pathway than in WT mice.

Finally, the above data illustrate how PCSK9 is dominant in the control of liver surface levels of LDLR, likely not reflecting the cellular need in cholesterol but rather regulating the clearance rate of circulating LDL by the liver.

Fig. 7. A high-cholesterol diet (HCD) abrogates the E2-specific regulation of surface LDLR in the liver of PCSK9 KO mice. A: LDLR immunohistochemistry was performed on 5 to 6 mice per sex and genotype. A representative picture is shown. B: PCSK9 ELISA in WT mice fed chow (n = 4–5) or a HCD (n = 7). C: LDLR and PCSK9 mRNA levels (n = 5–7). Averages normalized to that of the WT males are given. *P < 0.05; **P < 0.005; ***P < 0.0005. D: Representative Western blot analysis of the LDLR. LDLR band intensities were quantified by densitometry and normalized to that of β-actin, and WT male signal was set to 1. In C and D, the percentages of HCD versus chow diet values are shown.
Our data reveal that the absence of PCSK9, and thus of PCSK9-triggered LDLR degradation, leads to higher total LDLR protein levels in liver, pancreas, and small intestine extracts in both female and male mice (Figs. 1, 2). Immunohistological analyses, however, revealed a sex- and tissue-dependent subcellular distribution of the LDLR. In the absence of PCSK9, the LDLR labeling at the cell surface was dramatically higher in the liver and pancreatic islets of male, but not female, mice (Figs. 1C, 2A) and in the small intestine of female, but not male, mice (Fig. 2B). The latter observation was also true for the VLDLR, another target of PCSK9, whose surface labeling in perigonadal fat deposits was more strongly increased in KO female than male mice (Fig. 6) (22). We confirmed the above data by analyzing liver homogenates and their corresponding PM fractions. In male mice, the same 3-fold increase in LDLR protein in KO homogenates and PM fractions was observed. In contrast, the 4.6-fold LDLR increase in KO female homogenates had no repercussion on the LDLR content of their PM fractions (Fig. 3).

LDLRAP1 (ARH), Dab2, and IDOL are known to regulate LDLR endocytosis and/or the degradation of cell surface LDLR. However, none of them was regulated by E2 (supplementary Fig. 2). In contrast, we were able to show that the E2-specific LDLR subcellular distribution was lost upon an excess of dietary cholesterol because LDLR accumulated at the hepatocyte cell surface of KO male and female mice (Fig. 7). Estrogens usually bind estrogen receptors, namely ERα, that activate or repress transcription through direct interaction with estrogen response elements in promoters or interaction with other transcription factors. They were shown to alter the cyto-architectural properties of breast cancer cells, in which genes implicated in cell adhesion/ECM, cytoskeleton/cell architecture, or signal transduction were down-regulated rather than increased by E2 (43). As possible mechanisms, E2 may inhibit LDLR sorting to the cell surface or may shorten its residence time therein, leading to lower cell surface LDLR levels at a given time in KO female hepatocytes. It has been reported that, depending on the availability of cotranscriptional factors, E2 may exert cell-specific activities (44). Whether in the intestine or adipose tissue compared with liver E2 affects another target or has a modified activity remains to be determined. Microarray profiling of the livers from untreated KO female mice versus ovariectomized and/or cholesterol-fed mice may allow the identification of differentially regulated candidate genes.

Our work also suggests that PCSK9 and ovarian estrogens act synergistically to minimize fat accumulation in the mouse perigonadal pad (Fig. 5B). The separate loss of PCSK9 or ovaries resulted in modest fat accumulation, whereas their combined loss led to a 2.2-fold fat accumulation. Similarly, E2 supplementation was more efficient in the presence of PCSK9 to reduce perigonadal fat. Interestingly, although 15-fold higher E2 concentrations in OvxE2 mice had a similar impact on PCSK9, LDLR, or HMG-CoA-reductase mRNA levels as endogenous estrogens in sham mice, E2 supplementation had a greater impact than endogenous estrogens on the percentage of fat mass. E2 was reported to reduce visceral adiposity by increasing energy expenditure, decreasing inflammation, and reducing food intake via the regulation of orexigenic and anorexigenic peptides (for a review, see Ref. 36). On the other hand, PCSK9 seems to reduce visceral fat accumulation independently of the LDLR by promoting the degradation of the VLDLR in adipocytes, thereby limiting fatty acid internalization (22). Although further metabolic studies on the complementary roles of E2 and PCSK9 will be needed to elucidate the basis of their synergistic effects, the interconnection of their pathways seems evident. Whether a sustained treatment of hypercholesterolemic patients with a PCSK9 monoclonal antibody, especially in postmenopausal women, may favor visceral fat accumulation remains to be evaluated.

The sex-specific distribution of the LDLR was observed in KO mice only. Although we cannot rule out a role of PCSK9 deficiency in the setting of this regulation, our favored hypothesis is that the “eraser” effect of PCSK9 on cell surface LDLR or VLDLR is dominant and that its presence masks other mechanisms that regulate the surface levels of these two receptors.

What is the physiological relevance of these observations? Because cell surface labeling of the LDLR in the liver or islets of Langerhans was reported previously in PCSK9 KO male mice only (19, 21, 29), PCSK9 deficiency was thought to favor the accumulation of the LDLR at the cell surface of hepatocytes or β-cells and to result in an increased LDLR activity and, consequently, LDL depletion from circulation. Our data now show that PCSK9 KO female mice exhibit lower levels of surface LDLR than male mice in the liver. A lower cell surface liver LDLR activity would prolong the availability of all circulating LDLR-binding lipoproteins to extrahepatic tissues. However, we observed the same fat accumulation in KO male and female perigonadal fat and the same impact on adipocyte size (22). In addition, an extensive study that included male and female PCSK9 Y119X (KO) mice revealed that cholesterol homeostasis was well maintained in both sexes in the absence of PCSK9 (45), although a modest increase in LDL-cholesterol levels, which are as low as 5 mg/dl, is not easy to assess. Curiously, LDL mass measured by ELISA revealed a 35% drop in KO male mice but an unchanged value in KO female mice, indicating that the total mass of LDL is 37% higher in KO female mice than in KO male mice (supplementary Fig. 3). Future comparative analyses of the size, composition, and in vivo uptake of labeled lipoproteins in KO mice will be valuable. In this regard, if the estrogen-mediated regulation of LDLR surface levels that we observed in PCSK9 KO mice has its counterpart in humans and leads to a lower LDLR activity, we may expect a lesser impact of PCSK9 inhibitory
monoclonal antibodies on reducing LDL-cholesterol in premenopausal women than in men. No such evaluation according to sex is yet available but should emanate from the ongoing phase III clinical trials.

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