The proprotein convertase PCSK9 gene is the third locus implicated in familial hypercholesterolemia, emphasizing its role in cardiovascular diseases. Loss of function mutations and gene disruption of PCSK9 resulted in a higher clearance of plasma low density lipoprotein cholesterol, likely due to a reduced degradation of the liver low density lipoprotein receptor (LDLR). In this study, we show that two of the closest family members to LDLR are also PCSK9 targets. These include the very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) implicated in neuronal development and lipid metabolism. Our results show that wild type PCSK9 and more so its natural gain of function mutant D374Y can efficiently degrade the LDLR, VLDLR, and ApoER2 either following cellular co-expression or re-internalization of secreted human PCSK9. Such PCSK9-induced degradation does not require its catalytic activity. Membrane-bound PCSK9 chimeras enhanced the intracellular targeting of PCSK9 to late endosomes/lysosomes and resulted in a much more efficient degradation of the three receptors. We also demonstrate that the activity of PCSK9 and its binding affinity on VLDLR and ApoER2 does not depend on the presence of LDLR. Finally, in situ hybridization show close localization of PCSK9 mRNA expression to that of VLDLR in mouse postnatal day 1 cerebellum. Thus, this study demonstrates a more general effect of PCSK9 on the degradation of the LDLR family that emphasizes its major role in cholesterol and lipid homeostasis as well as brain development.

Familial hypercholesterolemia is mainly characterized by elevated plasma LDL cholesterol that is highly correlated with cardiovascular diseases (1). The main player in regulating the circulating cholesterol is the low density lipoprotein receptor (LDLR), which is expressed mostly in the liver. Recently, natural mutations in the proprotein convertase PCSK9 (2, 3) have been identified and associated with the third locus implicated in familial hypercholesterolemia (4–6). The major function of PCSK9 seems to be an enhancement of the degradation of the LDLR (7, 8) in acidic subcellular compartments (3), likely endosomes/lysosomes (9, 10). This can occur either via an extracellular endocytic route (11), or possibly by a direct cellular circuit not involving cell surface endocytosis of the LDLR (12). The gain of function PCSK9 mutations D374Y (13, 14) or D374H (15) have the highest impact on the development of hypercholesterolemia (16), likely through enhanced binding (17) and degradation of the LDLR (18, 19). The major binding site of LDLR to PCSK9 seems to reside within its first epidermal growth factor-like repeat namely EGF-A (20). Finally, it was recently suggested that the PCSK9-induced degradation of the cell surface LDLR does not require its proteolytic activity (21).

One of the unanswered questions is the target specificity of PCSK9, and it is not known, nor obvious, whether other members of the LDLR family are also affected by PCSK9. This family consists of structurally closely related transmembrane proteins: LRP1, LRP1b, megalin/LRP2, LDLR, very low density lipoprotein receptor (VLDLR), MEGF7/LRP4, LRP8/apolipoprotein E receptor 2 (apoER2) (22). Earlier studies revealed that LRP1 is not degraded by PCSK9 (3, 12). However, because primary sequence alignment revealed that the closest structural members to LDLR are VLDLR (59% identity) and ApoER2 (46% identity) (supplemental Fig. S1), we tested the potential degradation activity of PCSK9 on these two receptors.

Our data demonstrate that wild type PCSK9, and more so its natural mutant D374Y, enhance the degradation of VLDLR and ApoER2 in an LDLR- and catalytic activity-independent manner. Furthermore, the expression of either ApoER2 or VLDLR in CHO-A7 cells lacking endogenous LDLR enhanced the cellular association of exogenous PCSK9. Finally, we show that intracellular targeting of membrane-bound PCSK9 chimera accentuates their activity on the three receptors.
**Experimental Procedures**

**Sequence Alignment**—As shown in Fig. S1, the full-length sequence of ApoER2 (NP_150643), VLDLR (NP_003374), and LDLR (NP_000518) were aligned using the Multalin (23) and Genedoc software (National Resource for Biomedical Supercomputing; www.nrbsc.org).

**cDNAs and Cells**—Human PCSK9 and its mutant cDNAs were cloned into pIRES2-EGFP (Clontech) with or without a C-terminal V5 tag as described (4). The plasmids encoding for the ApoER2 and VLDLR were reported in (24). HEK293, Neuro2A, CHO-K1, and hepatic HepG2 cells (American Type Culture Collection, Manassas, VA) and HuH7 cells (a gift from François Jean, University of British Columbia) were routinely cultivated in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum. CHO-A7 (ldlA7; lacking the LDLR) and its parent CHO-WT were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum (Invitrogen) (25). These cells were also stably transfected with either cDNAs of empty vector pcDNA3, or recombinants of VLDLR and ApoER2. Stable pools (DNA3-A7; VLDLR-A7 and ApoER2-A7) were isolated by G418 (600 μg/ml) selection. The stable murine fibroblasts NIH 3T3 cells that express the cytoplasmic Dab1 protein and either ApoER2 (A+/D) or VLDLR (V−/D) were described in Ref. 24. Briefly, 3T3 cells were sequentially selected for expression of Dab1 (D) and for ApoER2 having the proline-rich cytoplasmic insert (A+) or for VLDLR (V−) that lack the o-glycosylation site. The cells containing a puromycin resistance were maintained at a concentration of 0.75 μg/ml.

**Conditioned Media**—HEK293 cells were transfected using Effectene transfection reagent (Qiagen) and kept for 24 h in serum-depleted media. The conditioned media were then transferred to 3T3 cells 6 or 24 h prior to analyses. For immunocytochemistry detection of the re-internalization assay, a final concentration of 10 μM NH₄Cl was added to the conditioned media.

**Biosynthetic Analysis**—HEK293 cells (2–4 × 10⁶) in 60-mm dishes were transiently transfected using Effectene (Qiagen) with 1.2 μg of wild type PCSK9-V5 (WT), PCSK9-V5-[TM-CT-Lamp1] (L1), PCSK9-V5-[TM-CT-LDLR] (LDLR), PCSK9-V5-[TM-CT-Ace2] (ACE2), or pIRE2 empty vector control (Ctl) in the presence or absence of co-transfected hLDLR cDNA. Two days post-transfection the cells were washed and pulse-labeled with 400 μCi/ml [³⁵S]Met + Cys (GE Healthcare) for 4 h (26). The cell lysates were immunoprecipitated with mAb:V5 (1:500) in buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 6.8, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl and 0.1% (v/v) SDS with a Complete Protease Inhibitor Mixture (Roche Applied Science). Proteins were separated by SDS-polyacrylamide gel electrophoresis (8% gels) and blotted on HyBond nitrocellulose membranes (GE Healthcare), which were blocked for 1 h in TBS-T (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 10% nonfat dry milk. Afterward, membranes were incubated overnight in 5% nonfat milk with the respective antibodies (Ab): ApoER2 (a23, 1:3000 (28), VLDLR (a74, 1:2000 (29), LDLR (1:5000, Abcam), PCSK9 (1:3000 (10), hepatocyte growth factor receptor (1:1000, Santa Cruz), ACE-2 (1:2000, R&D Systems), Lamp1 (1:2000 (30), and β-actin (1:3000, Sigma). Appropriate horseradish peroxidase-conjugated antibodies (1:10,000, Sigma) were used for detection with enhanced chemiluminescence using the ECL plus kit (GE Healthcare).

**Immunofluorescence and Confocal Microscopy**—At 48 h post-transfection, the cells were sequentially washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100/phosphate-buffered saline for 10 min, and incubated with 150 μg/ml glycine to stabilize the aldehyde. The cells were then incubated for 30 min with 1% bovine serum albumin (Fraction V, Sigma) containing 0.1% Triton X-100, followed by overnight incubation at 4 °C with rabbit polyclonal antibodies Ab:PCSK9 (1:1000), Ab: VLDLR (a74, 1:2000), Ab:ApoER2 (a23, 1:2000), and monoclonal Ab:V5 (1:1000, Invitrogen) in blocking solution with or without the late endosomes marker Ab:Cl-MPR (cation-independent mannose 6-phosphate receptor (Cl-MPR, 1:500, Abcam)). Afterward, the cells were incubated for 60 min with Alexa Fluor 647-conjugated goat anti-rabbit IgG and Alexa Fluor 555-conjugated goat anti-mouse IgG (both at 10 μg/ml; Molecular Probes) and mounted in 90% glycerol + 1% 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma). Immunofluorescence analyses were performed with a Zeiss LSM-510 confocal microscope coupled with a Nikon Eclipse TE2000-U laser-scanning microscope with 408-, 488-, and 543-nm laser lines. Images were processed with Adobe Photoshop CS2, version 9.0 (Adobe Systems).

**In Situ Hybridization (ISH) in Mouse**—For ISH, mouse sense and antisense cRNA probes coding for mouse PCSK9 (nucleotides 1197–2090, accession number NM_153565) (2) and mouse VLDLR (nucleotides 1193–2803; accession number NM_013703) or ApoER2 (nucleotides 2320–3030; accession number NM_004631) were labeled with [³²P]UTP and [³⁵S]CTP (1,250 Ci/mmol; Amersham Biosciences), to obtain high specific activities of ~1000 Ci/mmol. Eight to 10-μm whole mouse cryosections obtained at day 1 after birth (P1) were fixed for 1 h in 4% formaldehyde and hybridized overnight at 55 °C as described (31). For autoradiography, the sections were dipped in photographic emulsion (NTB-2, Kodak), exposed for 6–12 days, developed in D19 solution (Kodak), and stained with hematoxylin and eosin.

**Results**

**VLDLR and ApoER2 Are Novel PCSK9 Targets**—Whereas LR1 exhibits a 40% identity to LDLR (3, 12), it was not degraded by wild type PCSK9 or its gain of function mutant S127R (3). Because the primary sequences of ApoER2 and VLDLR exhibit the highest identity and similarity to that of LDLR (46 and 49 and 59 and 65%; supplemental Fig. S1), it was of interest to assess whether PCSK9 may also enhance the degradation of these receptors. To test this hypothesis, HEK293
ApoER2, VLDLR, and LDLR either with an empty vector (Ctl) or PCSK9. Twenty-four hours later, we analyzed by Western blot the steady state levels of each receptor in total cell lysates. Clearly, as for the LDLR, the presence of PCSK9 resulted in a substantial decrease in the protein levels of both ApoER2 and VLDLR. As control, PCSK9 did not affect the amount of a transmembrane protein angiotensin-converting enzyme 2 (ACE-2) suggesting that the three receptors may share a common specific motif that is not present in ACE-2.

We then extended our observations by co-transfecting NIH 3T3 fibroblasts with VLDLR or ApoER2 either with an empty vector (Ctl) or with recombinant PCSK9. The data revealed that in HEK293 cells, PCSK9 enhances the degradation of both ApoER2 and VLDLR both by co-expression or secretion of PCSK9 resulted in a substantial decrease in the protein levels of both ApoER2 and VLDLR. As control, PCSK9 did not affect the amount of a transmembrane protein angiotensin-converting enzyme 2 (ACE-2) suggesting that the three receptors may share a common specific motif that is not present in ACE-2.

Using HuH7 cells, we then decided to study the subcellular localization of trans-expressed wild type PCSK9 (t-PCSK9-wt, Fig. 2). We first expressed in HuH7 cells the full-length PCSK9-wt and its natural mutant PCSK9-D374Y. Our confocal microscopy analyses show that both constructs (PCSK9-wt and its natural mutant PCSK9-D374Y) are present in the late-endosomes/lysosomes compartment based on their co-localization with the Cl-MPR marker (Fig. 2B, red). We then expressed in trans V5-proPC9 with ΔproPC9-wt and show that both molecules co-localized in specific compartments within the cells in a similar fashion to PCSK9 (V5 and PCSK9 immunoreactivity), likely representing late-endosomes/lysosomes. In fact, our data suggest that as compared with the full-length PCSK9, trans-expression (proPC9 + ΔproPC9-wt) resulted in a similar subcellular localization and degradation function on the three receptors, in a PCSK9 catalytic activity-independent fashion.
PCSK9 Enhances the Degradation of VLDLR and ApoER2

To study the binding affinity of PCSK9 on either ApoER2 or VLDLR and to define whether the effect of exogenous PCSK9 on these receptors is also independent of LDLR, we generated CHO-A7 pools that stably express an empty vector (DNA3-A7, ApoER2-A7, VLDLR-A7). We then incubated overnight these cells with media obtained from CHO-A7 pools that stably express an empty vector (DNA3-A7, ApoER2-A7, VLDLR-A7). We then addressed the question if the LDLR could be limiting for ApoER2 and VLDLR degradation by exogenous PCSK9. For this purpose, we transiently expressed an empty vector or recombinant human LDLR in our CHO-A7 stable cell lines (DNA3-A7, ApoER2-A7, VLDLR-A7, Fig. 4). Twenty-four hours post-transfection, cells were incubated overnight with a conditioned media derived from HEK293 cells transiently expressing an empty vector (Ctl), PCSK9-wt, or PCSK9-D374Y (Fig. 4A). Clearly, in all cell lines, exogenous PCSK9-wt and its D374Y mutant resulted in efficient degradation of the transfected LDLR independent from the presence of either ApoER2 or VLDLR (Fig. 4, B–D). Our results also suggested that LDLR expression seems to compete for VLDLR degradation by PCSK9 with little or no effect on either receptor, exogenous PCSK9-D374Y is able to partially reduce the levels of ApoER2 and more so VLDLR, even though similar amounts were associated with both cells.

Because the degradation of ApoER2 and VLDLR by co-expressed PCSK9 is LDLR-independent, we then addressed the question if the LDLR could be limiting for ApoER2 and VLDLR degradation by exogenous PCSK9. For this purpose, we transiently expressed an empty vector or recombinant human LDLR in our CHO-A7 stable cell lines (DNA3-A7, ApoER2-A7, VLDLR-A7, Fig. 4). Twenty-four hours post-transfection, cells were incubated overnight with a conditioned media derived from HEK293 cells transiently expressing an empty vector (Ctl), PCSK9-wt, or PCSK9-D374Y (Fig. 4A). Clearly, in all cell lines, exogenous PCSK9-wt and its D374Y mutant resulted in efficient degradation of the transfected LDLR independent from the presence of either ApoER2 or VLDLR (Fig. 4, B–D). Our results also suggested that LDLR expression seems to compete for VLDLR degradation by PCSK9 with little or no effect on either receptor, exogenous PCSK9-D374Y is able to partially reduce the levels of ApoER2 and more so VLDLR, even though similar amounts were associated with both cells.

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**PCSK9 Enhances the Degradation of VLDLR and ApoER2**

**FIGURE 3. LDLR-independent degradation of ApoER2 and VLDLR by PCSK9.**

A. CHO-A7 cells were transiently co-transfected with ApoER2 or VLDLR either with an empty vector (pIREs; Ctl) or different PCSK9 constructs (wt or D374Y). B. Western blot using the PCSK9 antibody Ab:P9 (10) of conditioned media derived from HEK293 cells expressing empty vector, PCSK9 (in duplicate), or its D374Y mutant (in duplicate). C. Western blot of either DNA3-A7 or ApoER2-A7 cells incubated overnight with media obtained in B using either Ab:ApoER2 or Ab:P9. D. Western blot of either DNA3-A7 or VLDLR-A7 cells incubated overnight with media obtained in B using either Ab:VLDLR or Ab:P9. The levels of cellular β-actin are shown as a measure of gel loading. Cellular association of exogenous PCSK9-wt and PCSK9-D374Y on ApoER2-A7 and VLDLR-A7 are shown in duplicates (C and D).

**FIGURE 4. Additive binding of exogenous PCSK9 on CHO-A7 cells co-expressing LDLR with either ApoER2 or VLDLR.**

A. HEK293 cells were transiently transfected with either an empty vector (Ctl), PCSK9-wt, or PCSK9-D374Y. The levels of secreted PCSK9 are shown by Western blot analysis using the antibody Ab:P9 (10). B–D, DNA3-A7 (B), ApoER2-A7 (C), and VLDLR-A7 (D) cells were transfected with either an empty vector or with LDLR (+ LDLR). Twenty-four hours post-transfection, cells were incubated overnight with different conditioned media obtained in A. The steady state levels of LDLR and respective receptors (ApoER2 or VLDLR) were analyzed by Western blot. Cellular association of exogenous PCSK9 was also analyzed by Western blot for all cell lines (B–D). The levels of cellular β-actin are shown as a measure of gel loading. Similar data were obtained in a separate duplicate experiment (not shown).

Efficacy of PCSK9 to enhance the degradation of the three receptors versus its gain of function mutant D374Y (13, 14, 16), which is known to degrade (18, 19) and bind (17) LDLR, ApoER2, and VLDLR much more efficiently. Western blot analyses using our PCSK9 antibody (10) revealed that the expression and secretion levels of both untagged PCSK9 and its D374Y mutant are similar in transiently transfected HEK293 cells (Fig. 5A). We then used these conditioned media as a source of PCSK9 as compared with control media (Ctl) obtained from HEK293 cells transiently transfected with an empty pIREs vector. Accordingly, NIH 3T3 cells stably expressing the adaptor protein Dab1 and either ApoER2 or VLDLR (24) were incubated with spent media for either 6 or 24 h, and the levels of the receptors in the respective lysates were analyzed by Western blot (Fig. 5B). The data show that the levels of ApoER2 and VLDLR are already reduced at 6 h by PCSK9 (36 and 50%) and more so by its D374Y mutant (66 and 72%), respectively. In the VLDLR expressing cells, at 6 h the levels of endogenous LDLR were also decreased by PCSK9 and its D374Y mutant (27 and 46%). The decreased levels of ApoER2, VLDLR, and LDLR were much more evident at 24 h postincubation, revealing a decrease of 43, 43, and 50% by PCSK9 and 84, 89, and 70% by the D374Y mutant, respectively. These data demonstrate that in NIH 3T3 cells both PCSK9 and its D374Y mutant effectively enhance the degradation of all three receptors, albeit with different efficiencies, with the VLDLR seemingly being the most susceptible to exogenous PCSK9 activity.

**Internalized PCSK9 Co-localizes with ApoER2 and VLDLR—**To define the cellular localization of the internalized PCSK9, we incubated the stable NIH 3T3 cells (ApoER2, NIH 3T3 A+/D; VLDLR, NIH 3T3 V−/D) with conditioned media provided by HEK293 cells expressing either an empty vector (pIREs-V5) or recombinant PCSK9-V5 (Fig. 6). To prevent degradation of the receptors in the acidic endosomes/lysosomes (10), we incubated the NIH 3T3 cells with conditioned media containing both PCSK9 and 10 mM of the alkalinizing agent NH₄Cl, which was previously shown to block the PCSK9-induced degradation of the LDLR (3). Clearly, internalized PCSK9 co-localizes with both ApoER2 (Fig. 6A) and VLDLR (Fig. 6B) in perinuclear and punctate structures, reminiscent of those observed with the LDLR (3). Thus, the exogenous PCSK9-induced receptor degradation observed in NIH 3T3 cells expressing either VLDLR or ApoER2 (Fig. 5B) may occur intracellularly and that the cytosolic adaptor Dab1 seems to play a major role in this process, as for ARH that was reported to be important for LDLR degradation by PCSK9 (11).

**Membrane-bound PCSK9 Chimeras Are More Effective in Enhancing the Degradation of ApoER2, VLDLR, and LDLR in Acidic Compartments—**We previously reported that C-terminal fusion of proteins to the transmembrane-cytosolic tail of the

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**Note:** The text is a continuation of the previously provided information and may not be complete or may require further context for full comprehension. The images and figures are placeholders for actual visual content that would typically accompany the text.
lysosomal-associated membrane protein Lamp1 results in direct sorting of the tagged protein toward endosomal/lysosomal compartments (10, 30, 34). This approach led to the realization that such chimeras could drag partner proteins toward the endosomal/lysosomal degradation pathway. For example, expression of integrin α9/β2-Lamp1 or tissue inhibitor of metalloproteases TIMP-2-Lamp1 resulted in the degradation of integrin αvβ5 (30) or the proprotein convertase PC5 (34), respectively. The data presented in Fig. 5B showed that only a small percentage (≈1%) of extracellular PCSK9 actually re-enters the cells and those in Fig. 6 suggested that cytosolic proteins may be necessary for efficient endocytosis and degradation of the receptors by exogenous PCSK9. We thus hypothesized that a chimera of PCSK9 that can no longer exit from the cell, but that effectively sorts to endosomes/lysosomes might be an efficient carrier of its partner proteins LDLR, ApoER2, and VLDLR into these degradation compartments. Accordingly, we designed three type-I membrane-bound PCSK9-V5 chimeras containing at their C terminus the transmembrane-cytosolic tail of Lamp1, LDLR, or ACE-2 (35) (Fig. 7A). We then tested the efficacy of each chimera in enhancing the degradation of the LDLR by biosynthetic analysis in HEK293 cells co-transfected with PCSK9 or its chimeras in the presence (+) or absence (−) of LDLR (Fig. 7B, Cells top). The data show that all three membrane-bound PCSK9 chimeras drastically enhance the ability of PCSK9 to increase the intracellular degradation of LDLR, with the Lamp1 and ACE-2 chimera being the most effective. We also noted a drastic decrease in the shedding of the membrane-bound PCSK9 into the media (Fig. 7B, Media in lower panel) and that the metalloprotease-induced shedding of the LDLR resulting in a secreted soluble form (10) is also mostly prevented in all co-expression situations (Fig. 7B, Media in top panel).

Immunocytochemical analysis of HuH7 cells transiently transfected with the above three chimeras, clearly showed that PCSK9 resulting from each construct can enter endosomes/lysosomes, as evidenced by its co-localization with late endosomes/lysosomes marker CL-MPR (10) (Fig. S2). Note that the cell surface localization of PCSK9-ACE-2 and PCSK9-LDLR is much more evident than that of PCSK9-L1, which is more present in the degradative endosomes/lysosomes. We
also analyzed the remaining levels of LDLR by cell fluorescence-activated cellular sorting analyses, and found that the PCSK9-L1 chimera is much more effective in reducing the cell surface LDLR in HuH7 compared with secreted PCSK9-wt or other chimeras (not shown).

Cellular studies on Lamp1 revealed that the protein is mostly targeted toward endosomes/lysosomes upon exit from the trans Golgi network, that only a small percentage transiently cycles to the cell surface, and that the sorting signal resides in its 11-amino acid long cytosolic tail (36). It is known that PCSK9 and LDLR co-localize in endosomes, acidic pH is needed for the degradation of LDLR (3, 9), and the PCSK9-LDLR complex is even tighter at acidic pH values (17). We thus reasoned that PCSK9-L1 may primarily function by a direct route from the trans Golgi network toward endosomes/lysosomes and that its effect on LDLR may also be abrogated at neutral pH values. Indeed, incubation of HuH7 cells with 10 mM NH$_4$Cl markedly diminished the effect of either PCSK9 or PCSK9-L1 on endogenous LDLR (Fig. 8A). This is in accord with the localization of PCSK9 with endogenous LDLR in early and late endosomes of HuH7 cells (10). As controls, we show that PCSK9 or PCSK9-L1 do not affect the levels of endogenous hepatocyte growth factor receptor. Furthermore, another chimera integrin β3-Lamp1 (β3-L1) (30) did not affect the levels of LDLR in the absence or presence of NH$_4$Cl (Fig. 8A). Immunocytochemical analysis at the confocal level revealed that in the presence of 10 mM NH$_4$Cl and compared with overexpressed PCSK9, the chimeric PCSK9-L1 completely co-localizes with the CI-MPR (Fig. 8B) (10). Thus, the enhanced activity of PCSK9-L1 correlates with its ability to efficiently sort to endosomes/lysosomes.

It was reported that PCSK9 induces the degradation of LDLR in a cell line-specific fashion. Thus, whereas quite active in enhancing the degradation of endogenous LDLR in HepG2 cells, it does not seem to work efficiently on LDLR in CHO cells (12), and PCSK9 degrades endogenous LDLR much more rapidly in HepG2 versus HEK293 cells (37). We thus examined whether PCSK9 and PCSK9-L1 could degrade the three receptors in a cell-specific fashion. We analyzed the effect of these constructs on the level of co-expressed ApoER2, VLDLR, and LDLR in Neuro2A, HuH7, and CHO-K1 cells (Fig. 9). The data show that in CHO-K1, and less so in HuH7 cells, PCSK9 enhances the degradation of VLDLR and ApoER2 with little effect on LDLR. In contrast, PCSK9 does not reduce the level of any of the three receptors in Neuro2A cells. Amazingly, in the three cell lines the chimeric PCSK9-L1 actively enhances the degradation of all three receptors. This suggests that the cell-specific dependence can be bypassed by the efficient intracellular targeting of PCSK9 to endosomes/lysosomes.

**DISCUSSION**

To further evaluate the potential activity of the convertase PCSK9 on other members of the LDLR family, we decided to
test its ability to enhance the degradation of ApoER2 and VLDLR, the closest family members to LDLR. Our results demonstrate that, in an LDLR-independent fashion, PCSK9 is able to affect the levels of both receptors either by its co-expression (Fig. 1) or cell-surface internalization (Figs. 3 and 4) and that its catalytic activity is not required (Fig. 2). We also show that exogenous addition of the gain-of-function D374Y Anglo-Saxon mutant is more active on enhancing the degradation of LDLR, ApoER2, and VLDLR (Fig. 5B). Using CHO-A7 cells (lacking endogenous LDLR) that stably express either ApoER2 or VLDLR, we demonstrate that the presence of both receptors increases the capacity of PCSK9, and more so of its natural mutant D374Y, to be associated with cells implicating that these receptors may well bind PCSK9 either directly or indirectly (Fig. 3). It was recently reported that the epidermal growth factor–like repeat A domain of LDLR binds directly PCSK9 (20). However, so far the LDLR-binding domain(s) of the secreted complex of PCSK9 and its inhibitory prosegment (2, 17) is unknown. The major importance of Asp374 in this interaction, especially when mutated to Tyr (D374Y), suggests that the exposed surface loop containing Asp374 (17) may participate in the interaction of PCSK9 with the LDLR.

A recent report suggested that upon 2 h incubation of exogenous PCSK9 with COS-M cells expressing LDLR or VLDLR, only LDLR-expressing cells bound PCSK9 (20). Although no data were presented concerning the PCSK9-induced degradation of LDLR or VLDLR in COS-M cells. In contrast, our data showed that an overnight incubation of exogenous PCSK9, and more so of its D374Y mutant, with CHO-A7 cells stably expressing either ApoER2 or VLDLR, resulted in an enhanced association of PCSK9 with these cells (Fig. 3, C and D). Thus, either PCSK9 has a higher affinity (2 h versus overnight incubation) for LDLR versus ApoER2 or VLDLR, or the interaction is cell-type dependent. We observed, however, an enhanced degradation of ApoER2 and VLDLR following their co-expression with PCSK9 and/or its chimeric PCSK9-L1 in six different cell types, namely HEK293, NIH 3T3, CHO-A7, CHO-K1, Neuro2A, and HuH7 cells. Furthermore, in COS-1 cells we could not demonstrate degradation of these receptors except with PCSK9-L1 (supplemental Fig. S3A). In addition, overnight incubation of exogenous PCSK9 or its D374Y mutant with COS-1 cells led to cellular association but did not result in the degradation of endogenous LDLR (supplemental Fig. S3B). Thus, we presume that the cell type-dependent efficacy of PCSK9-induced degradation of LDLR, ApoER2, or VLDLR may depend on specific cellular factors, such as ARH for LDLR and Dab1 for ApoER2 and VLDLR (40).

The effective degradation of the three receptors by the chimeric construct PCSK9-L1 clearly demonstrated that an efficient targeting of PCSK9 to the acidic endosomes/lysosomes (Fig. S2) maximizes its induced degrading function, even in CHO-A7 cells (not shown). Thus, independent of the LDLR, PCSK9 interacts with VLDLR and ApoER2 and drags them toward the intracellular degradative pathway.

Whereas knockdown of PCSK9 in zebrafish revealed a dramatic neuronal phenotype (41), this was not observed in either PCSK9...
knock-out mice (8) or in two women lacking functional PCSK9 (42, 43). Thus, it is possible that in mammals another gene may compensate partially for the absence of PCSK9 in brain. Because VLDLR and ApoER2 are known to exert their major effects during brain development (38), we also carefully analyzed the brains of our Pcsk9−/− mice and did not observe overt morphological defects.3 Because our work suggested that PCSK9 enhances the degradation of ApoER2 and VLDLR, it would be informative, however, to test possible developmental defects in mice overexpressing PCSK9 or its D374Y gain of function mutant in the cerebellum.

In conclusion, PCSK9 has now been shown to enhance the degradation of LDLR and its closest family members ApoER2 and VLDLR (this work), but not LR1P1 (3). The availability of the membrane-bound powerful PCSK9 chimeras will be very useful to define the panoply of other cellular proteins that could be affected by PCSK9.

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