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β-Estradiol results in a proprotein convertase subtilisin/kexin type 9-dependent increase in low-density lipoprotein receptor levels in human hepatic HuH7 cells

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The lower risk of coronary artery disease in premenopausal women than in men and postmenopausal women implicates sex steroids in cardioprotective processes. β-Estradiol upregulates liver low-density lipoprotein receptor (LDLR), which, in turn, decreases circulating levels of low-density lipoprotein, which is a risk factor for coronary artery disease. Conversely, LDLR protein is negatively regulated by proprotein convertase subtilisin/kexin type 9 (PCSK9). Herein, we investigated PCSK9 regulation by β-estradiol and its impact on LDLR in human hepatocarcinoma HuH7 cells grown in the presence or absence of β-estradiol. Immunoblot analysis showed upregulation of LDLR at 3 μM β-estradiol (140%), and the upregulation reached 220% at 10 μM β-estradiol; only at the latter dose was an increase in *LDLR* mRNA detected by qPCR, suggesting post-translational regulation of LDLR. No changes in PCSK9 mRNA or secreted protein levels were detected by qPCR or ELISA, respectively. β-estradiol-conditioned medium devoid of PCSK9 failed to upregulate LDLR. Similarly, *PCSK9* knockdown cells showed no upregulation of LDLR by β-estradiol. Together, these results indicate a requirement for PCSK9 in the β-estradiol-induced upregulation of LDLR. A radiolabeling assay showed a significant, dose-dependent decrease in the ratio of secreted phosphoPCSK9 to total secreted PCSK9 with increasing β-estradiol levels, suggesting a change in the functional state of PCSK9 in the presence of β-estradiol. Our results indicate that the protein upregulation of LDLR at subtranscriptionally effective doses of β-estradiol, and its supratranscriptional upregulation at 10 μM β-estradiol, occur through an extracellular PCSK9-dependent mechanism.

Introduction

Heart disease is the primary cause of mortality worldwide [1]; significant risk factors include gender, with males being more at risk than age-matched premenopausal females, a bias that is lost following menopause [2] and hypercholesteremia [3], a disorder of elevated circulating levels of low-density lipoprotein (LDL) cholesterol (LDLC). Indeed, these two risk factors are linked to a certain degree, as sex-specific hormones have been

Abbreviations
CV, coefficient of variation; DMEM, Dulbecco's modified Eagle's medium; ER, estrogen receptor; Idol, inducible degrader of low-density lipoprotein receptor; LDLC, low-density lipoprotein cholesterol; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; qPCR, quantitative PCR; SEM, standard error of the mean; βE2, β-estradiol.

implicated in cholesterol regulation [4,5]. In particular, the steroid hormone estrogen, which is produced primarily by the ovary, has been shown to have multiple cardioprotective effects [2]; these include decreasing circulating LDLC levels and increasing high-density lipoprotein cholesterol levels, decreasing circulating lipoprotein A levels, and preventing lipid peroxidation [6].

The liver LDL receptor (LDLR) is the major regulator of circulating LDLC levels, through uptake of LDL particles from the circulation. Following endocytosis, the interaction between LDL particles and LDLR is disrupted by the acidic pH of late endosomes, permitting the transport and the destruction of the LDL particle in the lysosome, and the recycling of LDLRs back to the cell surface. Transcriptionally, *LDLR* can be induced by estrogen through the estrogen receptor (ER) but not through the classic estrogen-responsive element site, which is absent in the *LDLR* promoter region [7]. Instead, ER induces transcription by its interaction with specific factor-1 sites [8]. Androgen does not increase the transcription of *LDLR*, and can attenuate the positive effects of estrogen [5,7].

Post-transcriptionally, liver LDLR levels are modified by the secreted glycoprotein proprotein convertase subtilisin/kexin type 9 (PCSK9) [9,10]. On the cell surface, extracellular interaction of LDLR with its escort protein, PCSK9, directs LDLR from the endocytic-recycling pathway towards the lysosomal pathway for degradation [9,11–13], although the exact mechanism is still not fully elucidated. Recently, a second type of post-transcriptional negative regulation of LDLR was identified [14]; inducible degrader of LDLR (Idol; Mir/Mylip) facilitates LDLR clathrin-independent internalization [15,16] and degradation by polyubiquitinating the receptor [14]. Regulation of PCSK9 and Idol are inversely related to the cholesterol level. An elevated cholesterol level inhibits the nuclear translocation of sterol regulatory element-binding protein, thus repressing both PCSK9 and LDLR expression, but can lead to liver X receptor activation, and in turn the activation of Idol. In a murine model, Sasaki *et al.* found that the hepatic overexpression of Idol resulted in an increase in circulating PCSK9 levels through a sterol regulatory element-binding protein/LDLR-dependent pathway [17].

Human population studies have shown that the post-transcriptional regulation of LDLR by PCSK9 is significant in terms of LDL homeostasis, whereby naturally occurring variants of PCSK9 cause changes in LDLR levels and hence affect circulating LDLC levels [18,19]. For instance, the PCSK9-D374Y variant binds 10-fold more to cell surface LDLR than wild-type PCSK9, shifting the equilibrium towards LDLR degradation and resulting in hypercholesterolemia [12,20]. In contrast to this, we have identified and characterized a PCSK9-Q152H variant in a French Canadian population that precludes PCSK9 secretion and results in significantly reduced levels of circulating LDLC (< 5th–14th percentile; age-matched and gender-matched) [21].

Rat models, wherein animals ingested or were injected with estradiol, showed that LDLR upregulation at the mRNA level was not sufficient to explain the exaggerated and significant increase in LDLR observed at the protein level [22]. Persson *et al.* concluded that decreased *PCSK9* transcription contributed to the downstream effect of elevated LDLR expression levels observed in rats in response to estradiol [23]. In humans, plasma PCSK9 levels are significantly higher in premenopausal, age-matched women than in men, despite significantly lower LDLC levels [24,25]. In addition, PCSK9 levels are elevated in postmenopausal [24] and pregnant women [26] as compared with premenopausal, nonpregnant women. Collectively, these findings implicate sex hormones in PCSK9 regulation.

Cell culture models have been utilized to evaluate the effects of estradiol on human LDLR, namely through the use of hepatocarcinoma HepG2 cells co-transfected with LDLR and the estrogen receptor [7,8,27]. As with the observed animal models, exogenous estradiol treatment resulted in elevated LDLR levels in ERα-overexpressing HepG2 cells. In the current study, we used the hepatocarcinoma HuH7 cell line to evaluate the effects of estradiol treatment on PCSK9 and LDLR, and compared these results with those obtained in HepG2 cells containing endogenously expressed receptors. Consistent with other systems, we found that LDLR protein levels were elevated in HuH7 cells following estradiol treatment, to a significantly greater extent than could be attributed to estradiol's transcriptional effects. However, and in contrast to the studies using rat models, we found that, in *PCSK9* knockdown HuH7 cells in β-estradiol (βE2)-primed PCSK9-deficient medium, upregulation of LDLR was dependent on the presence of PCSK9 rather than a reduction in *PCSK9* transcription. Furthermore, we found that estradiol treatment of HuH7 cells resulted in decreased phosphorylation of secreted PCSK9; HepG2 cells have a lower level of the phosphorylated form of secreted PCSK9 than HuH7 cells [28], and this was not further reduced by estradiol treatment. Together, these data indicate that estradiol-induced post-translational modification of PCSK9 may affect PCSK9 function, including the interaction of PCSK9 with LDLR. On the basis of these findings, we propose that an alternative, as yet undefined, mechanism exists for the regulation of LDLR by PCSK9 in the presence of estradiol.

Results

βE2 caused a dose-dependent increase in LDLR expression in HuH7 cells

Total cell lysates from HuH7 cells treated in serum and phenol red-free Dulbecco's modified Eagle's medium (DMEM) for 48 h with increasing subcytotoxic concentrations of βE2 were compared with control total cell lysates (cells treated with an equivalent volume of ethanol) by immunoblotting (Fig. 1). There were significant 1.4-fold, 1.7-fold and 2.2-fold increases in LDLR protein expression in cells treated with 3 μM, 5 μM and 10 μM βE2, respectively, as compared with control-treated cells (Fig. 1A). This upregulation of LDLR was prevented by pretreatment of cells with the estrogen receptor inhibitor fulvestrant (Sigma-Aldrich, Oakville, Canada) (Fig. 1A). To localize the upregulation of LDLR, cell surface proteins were biotinylated and enriched from cell lysates by streptavidin agarose immunoprecipation; membrane-enriched and membrane-depleted fractions were compared by immunoblotting, and this showed that LDLR levels were increased in both fractions as compared with transferrin receptor (membrane-enriched) and actin (membrane-depleted) (Fig. 1B). This was in concordance with the increase in the uptake of fluorescently labeled LDL upon stimulation with 10 μM βE2 (Fig. 1C), which was comparable to the increase in uptake in response to the positive control, compactin.

βE2 caused transcriptional upregulation of *LDLR*, but not of *PCSK9*

Transcriptional changes to *LDLR* in response to βE2 treatment of HuH7 cells were evaluated by quantitative PCR (qPCR). After 48 h, there was a significant 1.5-fold increase in *LDLR* transcription in cells treated with 10 μM βE2 relative to control-treated cells (Fig. 2A). In contrast to the dose-dependent elevated LDLR protein expression, there was no significant increase in *LDLR* mRNA at lower βE2 concentrations. A time course experiment was used to evaluate the effect of βE2 on *LDLR*, by the use of qPCR on samples treated with 10 μM βE2 for 0, 1, 2, 4, 6, 8, 24 and 48 h. As shown in Fig. 2B, the significant increase in mRNA expression of *LDLR* occurred as early as 8 h after the addition of βE2. Notably, βE2 caused upregulation of LDLR protein in HuH7 cells at a dose (3 μM) lower than that required for its transcriptional upregulation (10 μM). Also, LDLR protein was upregulated at levels greater (2.2-fold at 10 μM) than that explained by its transcriptional regulation (1.5 at 10 μM). These data imply that, as in the rat model

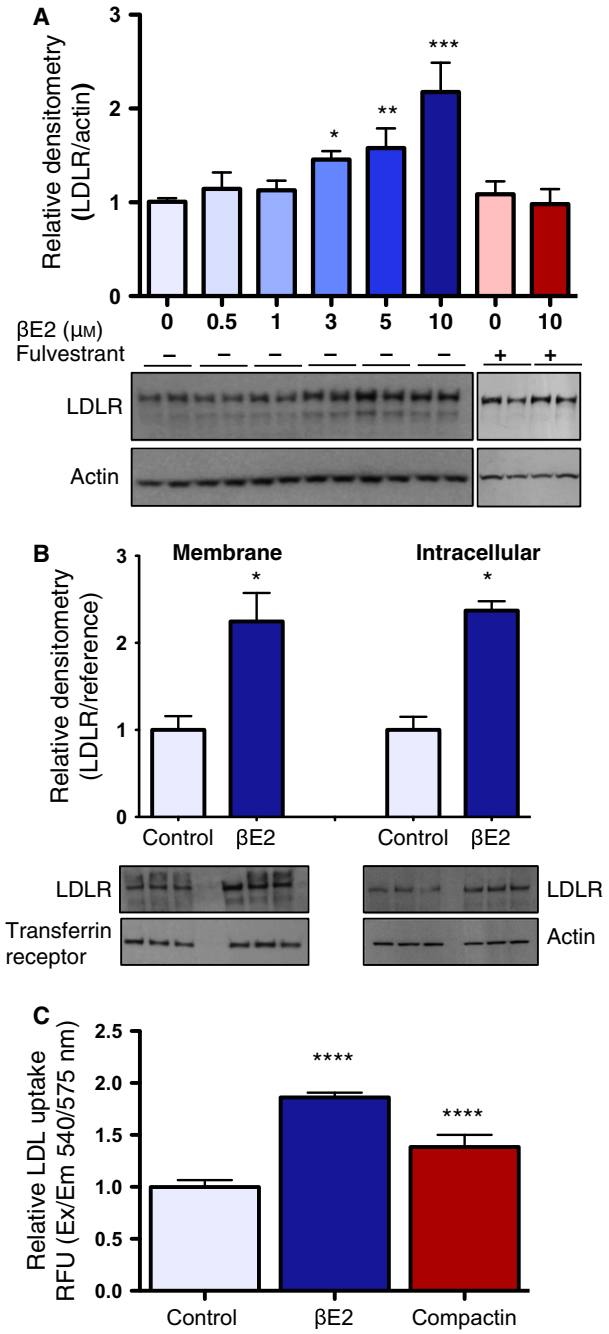

Fig. 1. HuH7 cells incubated with βE2 show a dose-dependent increase in LDLR protein levels. Human hepatocarcinoma HuH7 cells were grown in the presence of increasing concentrations of βE2 (μM) for 48 h. (A, B) Representative immunoblot and densitometry analysis of (A) total cell lysates and (B) membrane-enriched fractions indicate that LDLR levels were increased both intracellularly and at the cell surface in response to βE2. (C) Uptake of fluorescently labeled LDL by HuH7 cells in response to βE2. Data, normalized to control, represent the mean ± SEM; $n \geq 3$ experiments, each performed in triplicate. *$P < 0.05$, ** $P < 0.01$, ***$P < 0.005$, ****$P < 0.0005$. Em, emission; Ex, excitation; RFU, relative fluorescence units.

[23], LDLR in human HuH7 cells is also regulated post-transcriptionally. We assessed whether this was attributable to transcriptional downregulation of *PCSK9*; there was no significant change in *PCSK9* mRNA expression following βE2 stimulation as compared with vehicle-treated cells (Fig. 2C).

βE2 did not affect secreted PCSK9 levels

Extracellular, secreted PCSK9 has been shown to significantly affect the protein levels of LDLR in cell and animal studies [29–31]. Therefore, we used quantitative PCSK9 ELISA (Cyclex, Nagano, Japan) of conditioned medium to assess whether βE2 affected the levels of PCSK9 secreted from HuH7 cells. There was no significant change in the level of secreted PCSK9 following βE2 stimulation as compared with vehicle-treated cells (Fig. 2D).

PCSK9 levels were not affected by βE2 stimulation, but extracellular PCSK9 was necessary for its upregulation of LDLR in HuH7 cells

To further evaluate the role of secreted PCSK9 in the response to βE2 treatment, we generated *PCSK9* knockdown cells by using small hairpin shPCSK9. The knockdown of *PCSK9* was assessed at the gene level by real-time RT-qPCR (Fig. 3A) and at the protein level by PCSK9 ELISA (Fig. 3B). The level of *PCSK9* mRNA was decreased by 55%, and this was reflected by a 65% decrease in the level of secreted PCSK9. We confirmed the specificity of the shRNA by real-time RT-qPCR of *LDLR* mRNA, and found no effect on LDLR in these stable shPCSK9 knockdown pool cells (Fig. 3A). The reduction in PCSK9 levels resulted in an increase in LDLR levels in shPCSK9 cells as compared with nontarget controls, however, further upregulation of LDLR was still possible, as observed from compactin stimulation (Fig. 3C).

Consistent with Fig. 1, nontarget PCSK9 HuH7 cells showed a significant increase in LDLR levels in response to βE2 (Fig. 4A). In contrast to this, there was no significant increase in LDLR levels of targeted shPCSK9 knockdown βE2-treated cells as compared with vehicle-treated shPCSK9 knockdown cells (Fig. 4A). Although the shPCSK9 cells were still capable of fully upregulating LDLR, as shown by stimulation with compactin (Fig. 3C), these results suggest that the supratranscriptional upregulation of LDLR in response to βE2 is PCSK9-dependent. When spent media from the nontarget and shPCSK9 knockdown

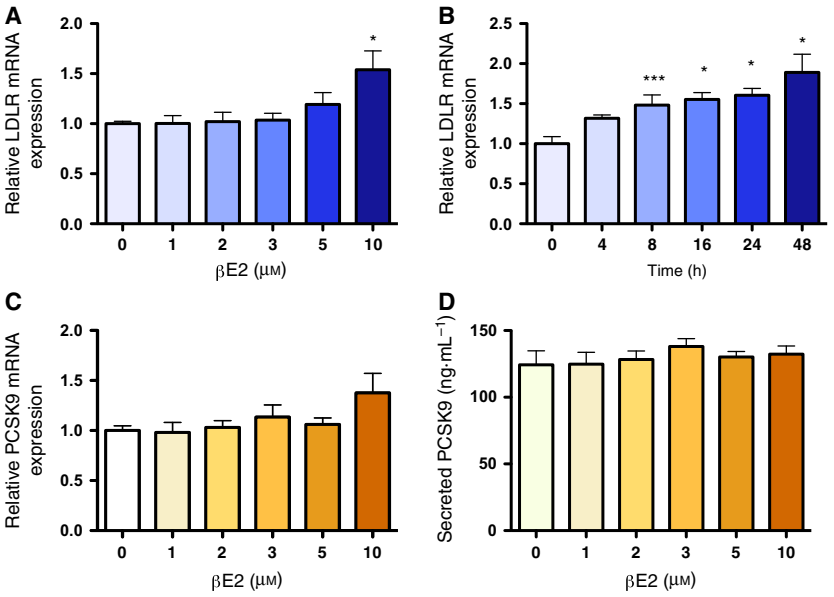

Fig. 2. The level of LDLR mRNA, but not of PCSK9 mRNA, is increased in HuH7 cells incubated with 10 μM βE2. (A–C) Gene expression was evaluated in HuH7 cells treated with (A) increasing concentrations of control or βE2 for 48 h, or (B) 10 μM βE2 at time points ranging from 0 h to 48 h. A significant increase in *LDLR* transcription at 48 h was observed only with 10 μM βE2, but could be observed as early as 8 h following βE2 addition to cells; in contrast, (C) mRNA levels of PCSK9 were unchanged following βE2 treatment. (D) Levels of secreted PCSK9 were measured by ELISA, and no significant change was observed in HuH7 media in response to βE2 treatment. Data, normalized to control, represent the mean ± SEM; $n = 3$ experiments, each performed in triplicate. $^*P < 0.05$, $^{***}P < 0.005$.

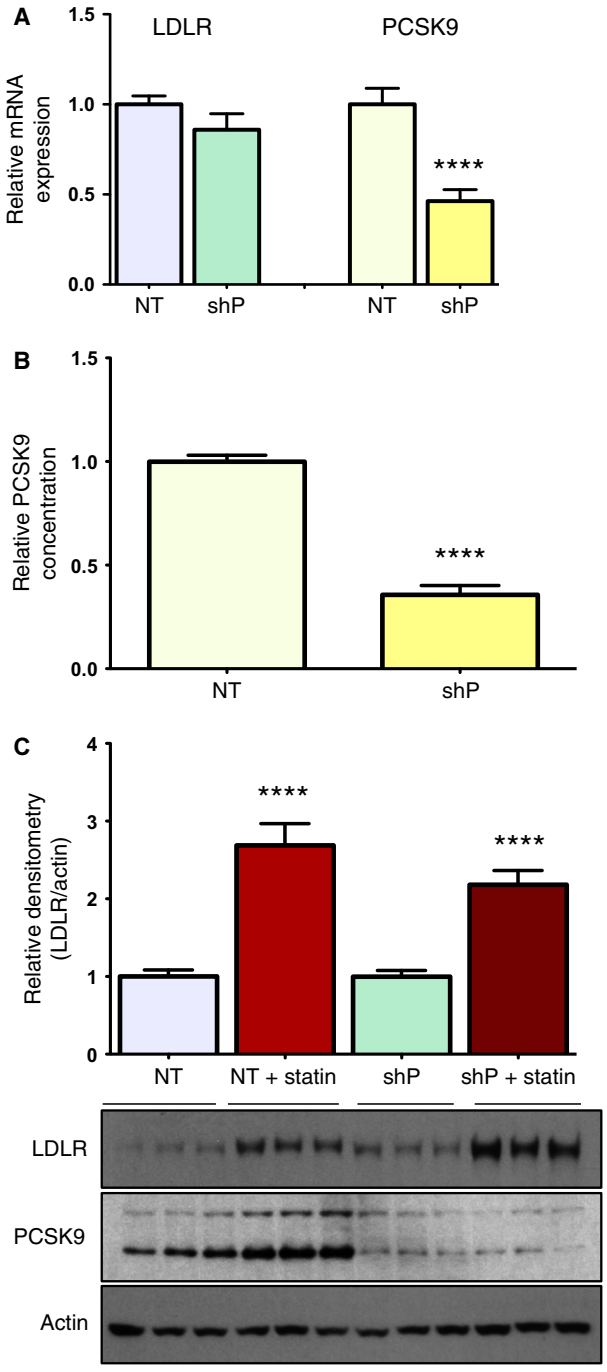

Fig. 3. Assessment of shRNA knockdown of PCSK9 in HuH7 cells. PCSK9 knockdown was evaluated in HuH7 cells that were transfected with nontarget scrambled shRNA (NT) or with PCSK9-targeted shRNA (shP). (A) The specificity and level of knockdown was assessed at the RNA level by qPCR, with primers for LDLR and PCSK9, respectively. (B) The level of knockdown was assessed at the secreted protein level by PCSK9 ELISA of cell-conditioned media, relative to the nontarget conditioned media. (C) NT or shP cells were incubated in triplicate in serum-free media for 24 h before the addition of vehicle or 10 μM compactin for another 24 h. LDLR levels in total cell lysates were (C) evaluated by immunoblotting; the resultant densitometry results are shown relative to vehicle-treated cells, and indicate that shP cells are as responsive to statins as their NT counterparts. $n = 3$ experiments. **** $P < 0.0005$.

cells were transferred onto naïve HuH7 cells for 24 h, a similar pattern of LDLR upregulation response was observed (Fig. 4B), although only the PCSK9-depleted control media resulted in elevated levels of LDLR as compared with the nondepleted control.

To evaluate whether the presence of PCSK9 in βE2-conditioned media was necessary for the supratranscriptional response in terms of upregulated LDLR protein, we carried out a βE2-conditioned medium transfer experiment in the presence and absence of secreted PCSK9. Media from cells stimulated with βE2 or control were stripped of PCSK9 by specific immunoprecipitation (with antibody against PCSK9/protein A/G agarose) or not (with protein A/G agarose as control), as detailed below. Immunoblotting of source cells confirmed upregulation of LDLR in response to βE2 (results not shown). Furthermore, ELISA of source-conditioned media before and after immunoprecipitation confirmed removal of 100% of PCSK9 prior to recipient incubation (results not shown). Transfer of βE2-conditioned media containing PCSK9 significantly upregulated LDLR (1.9-fold) in comparison with media transferred from vehicle-treated cells (Fig. 5A). In contrast, the level of LDLR in recipient cells incubated with PCSK9-depleted βE2-treated media was significantly lower than in the counterparts with PCSK9 present (Fig. 5A). Therefore, despite the presence of PCSK9 in source-conditioned media, it appears that βE2 treatment post-translationally affects PCSK9 – through modification, protein interaction, or a combination thereof – to attenuate the action of PCSK9 on LDLR. Furthermore, the effect of βE2 on LDLR upregulation was lost upon PCSK9 depletion of source-conditioned media, implicating a role for PCSK9 in its upregulation in this system.

Like HuH7 cells, HepG2 cells are hepatocarcinoma cells that express PCSK9, ERα, and LDLR, though the expression levels are 30%, 50% and 1.5-fold, respectively, of those in HuH7 cells (results not shown). We found that HepG2 cells expressing endogenous levels of LDLR and ER responded to estradiol treatment with a nonsignificant increase in LDLR expression (Fig. 5B); consistent with this, we observed no increase in LDL uptake by estradiol-stimulated

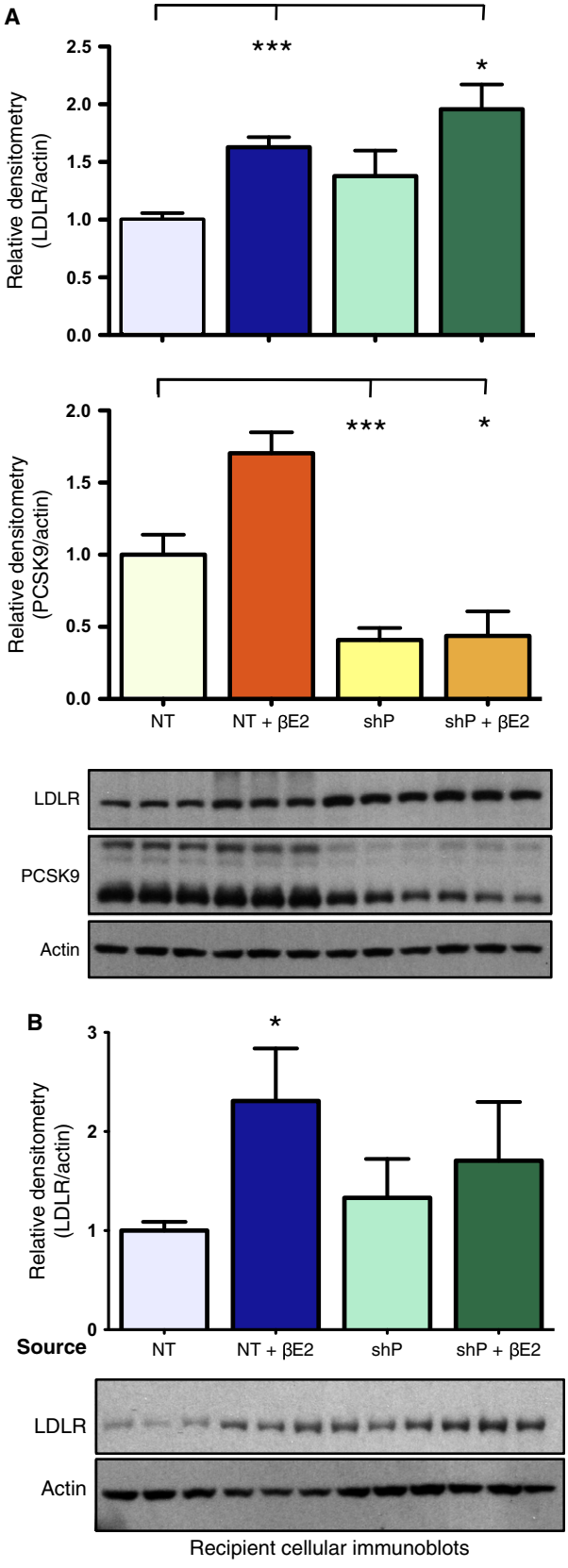

Fig. 4. LDLR levels are increased following βE2 treatment of nontarget but not shPCSK9 HuH7 cells. HuH7 cells transfected with nontarget scrambled shRNA (NT) or with PCSK9-targeted shRNA (shP) were grown in the presence of 10 μM βE2 for 48 h. (A) Representative immunoblotting and densitometry of total cell lysates indicate that LDLR levels were increased in NT cells but not in shP βE2-treated cells, indicating a requirement for PCSK9 in the βE2-induced upregulation of LDLR. (B) Conditioned medium of NT or shP cells treated with vehicle or βE2 was then transferred to naïve HuH7 cells for 24 h of incubation, and the recipient HuH7 cell lysates were analyzed by immunoblotting. Data, normalized to NT control for (A) and (B), represent the mean ± SEM; $n = 3$ experiments, each performed in triplicate. $^{*}P < 0.05$, $^{***}P < 0.005$.

HepG2 cells. Conversely, compactin-stimulated HepG2 cells did show an increase in LDL uptake (Fig. 5C). An additional media transfer experiment was performed with HuH7 recipient cells, this time with HepG2-treated source media. As in the HuH7 source media transfer experiment (Fig. 5A), media treated with βE2 and then depleted of PCSK9 resulted in a significant decrease in LDLR levels in recipient cells as compared with both PCSK9-present or control media-treated cell lysates (Fig. 5D). In contrast to what is seen in Fig. 5A, cells that were recipients of HepG2 control media depleted of PCSK9 showed a decrease in LDLR level, although the levels was not significantly different from that in the control.

βE2 affects the molecular form of secreted PCSK9

The above results suggest a βE2-induced shift in secreted PCSK9 in terms of its regulation of LDLR levels. We previously reported that PCSK9 was phosphorylated on its propeptide at Ser47 and in its Cys-rich and His-rich domain at Ser688 [28]. We also showed that prodomain phosphorylation was decreased by two PCSK9 variants that are associated with its 'loss of function' against LDLR and low LDLC levels, namely A53V [32] and R46L [32, 33], the mutations in which occur in proximity to the phosphoSer47 site. Furthermore, we showed that 70% of PCSK9 secreted by HuH7 cells is phosphorylated, which is in contrast to that secreted by HepG2 cells, the phosphorylation level of which is only 54% [28]. To evaluate whether βE2 affected PCSK9 phosphorylation, conditioned media from differentially radiolabeled HuH7 or HepG2 cells treated with βE2 were immunoprecipitated, and then both total PCSK9 ([^{35}S] Cys/Met radiolabeling; Fig. 6A,D) and phosphorylated PCSK9 ([^{32}P]orthophosphate radiolabeling; Fig. 6B,E) were quantified. As a percentage of the

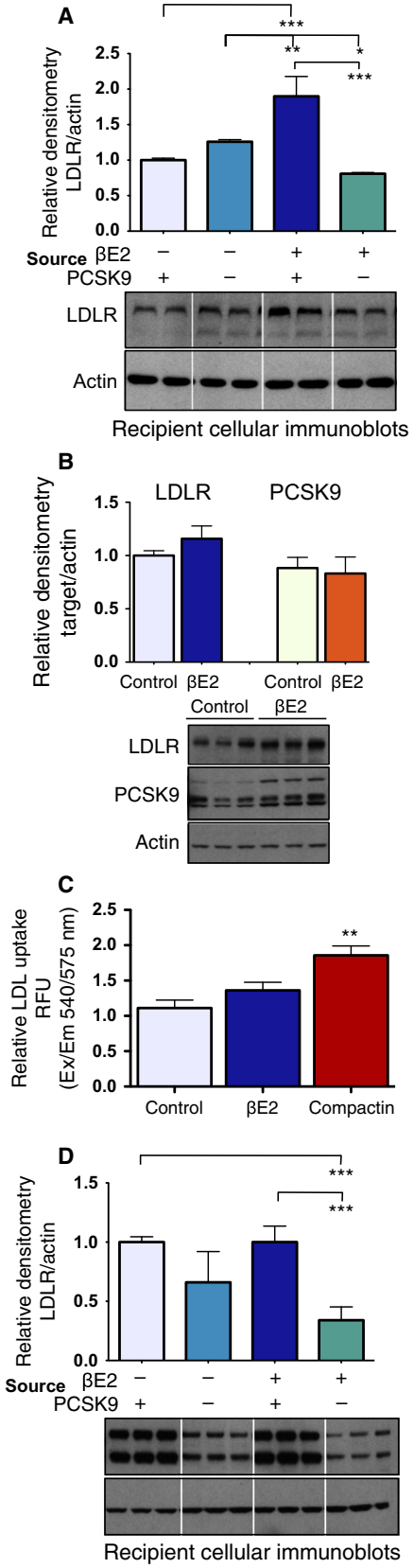

Fig. 5. Extracellular PCSK9 depletion eliminates LDLR upregulation by 10 μM βE2 treatment of HuH7 cells. Conditioned medium collected from control or 10 μM βE2-treated HuH7 (source) cells was immunoprecipated with protein A/G (control) or antibody against PCSK9, and used for the growth of fresh HuH7 cells for 24 h (recipient) cells. (A) Immunoblotting and densitometry of total cell lysates of recipient cells are shown. In comparison with control (lanes 1 and 2), LDLR levels were not significantly changed in recipient cells grown in PCSK9-depleted media (lanes 3 and 4; lanes 7 and 8), but LDLR levels were elevated in cells grown in media from cells that were βE2-treated (lanes 5 and 6). The removal of PCSK9 from βE2-treated cell media caused a decrease in LDLR levels (lanes 7 and 8) as compared with recipients of βE2-treated cell media containing PCSK9. (B) HepG2 cells were treated for 48 h with 10 μM βE2, and cell lysates were evaluated by immunoblotting and densitometry. (C) Uptake of fluorescently labeled LDL by HepG2 cells following treatment with 10 μM βE2 or 10 μM compactin is shown, and indicates upregulation of LDLR only by the statin. (D) Conditioned medium collected from control or 10 μM βE2-treated HepG2 (source) cells was immunoprecipated with protein A/G (control) or antibody against PCSK9, and used for the growth of fresh HuH7 (recipient) cells for 24 h. Immunoblotting and densitometry of total cell lysates of recipient cells are shown. Data, normalized to control, represent the mean ± SEM; $n > 3$ experiments, each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$. Em, emission; Ex, excitation; RFU, relative fluorescence units.

total secreted PCSK9, there was a dose-dependent decrease in the level of phosphorylated PCSK9 secreted by HuH7 cells in response to βE2 (Fig. 6C). There were significant reductions in phosphorylated PCSK9: 23.8%, 37.6% and 66.1% of total PCSK9 at 1 μM, 5 μM and 10 μM βE2, respectively. In contrast, 10 μM simvastatin, which has been previously shown to upregulate PCSK9 [25], increased the level of secreted PCSK9 in this analysis, as shown by [^{35}S]Cys/Met radiolabeling (Fig. 6A), without affecting its level of phosphorylation (Fig. 6B,C). Furthermore, fenofibrate, which has previously been shown not to change the level of secreted PCSK9 in cell culture [25], did not affect the level of phosphorylated PCSK9 (Fig. 6C). HepG2 cells treated with 10 μM βE2 showed a nonsignificant decrease of 29% in phosphorylated PCSK9 (Fig. 6F).

Discussion

In this article, we present findings indicating that PCSK9 is required for supratranscriptional upregulation of LDLR by βE2 in human hepatic HuH7 cells, and that this may be related to the phosphorylation state of secreted PCSK9. On the basis of our results,

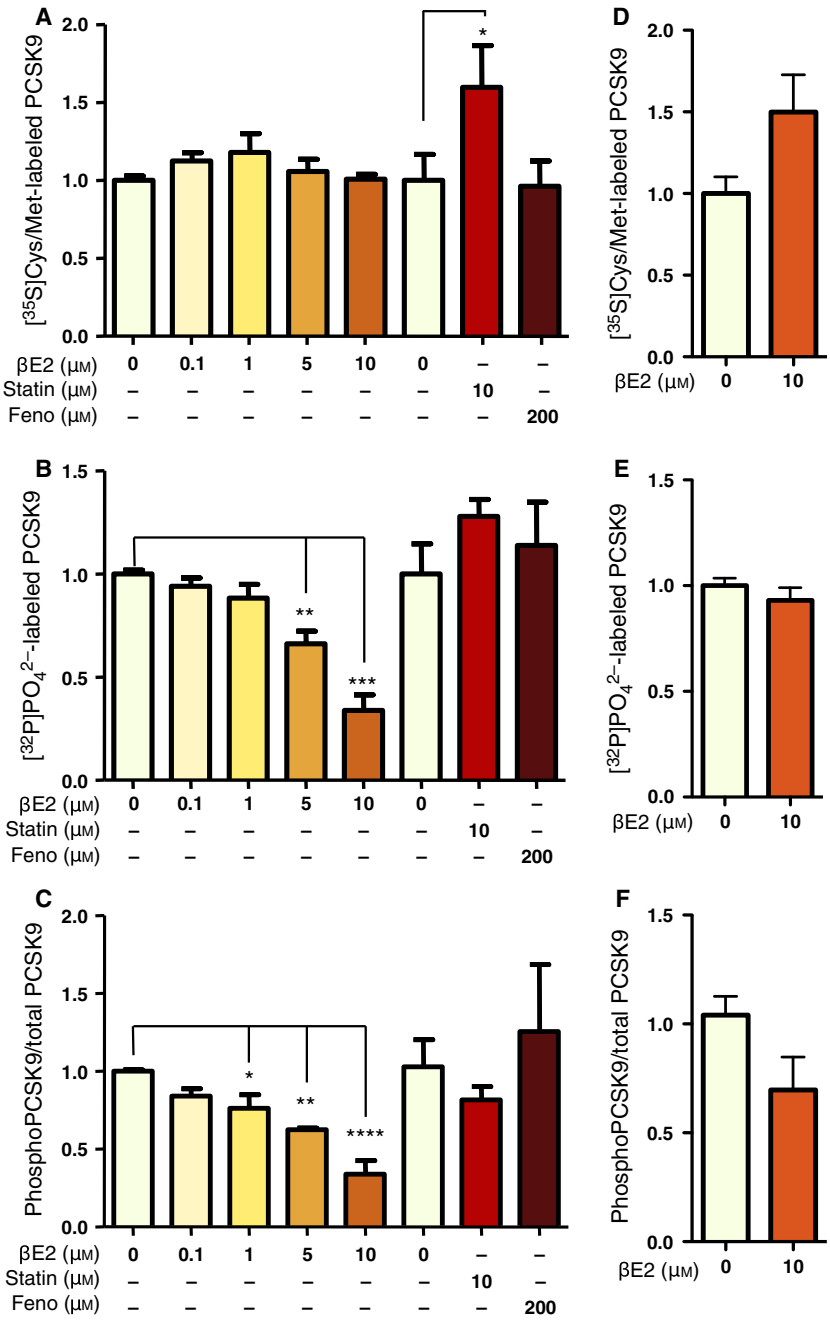

Fig. 6. The level of phosphorylated PCSK9 is decreased in βE2-treated HuH7, but not HepG2, cell-conditioned media. HuH7 (A–C) and HepG2 (D–F) cells were treated with βE2 at the concentrations indicated. Following immunoprecipitation from conditioned media, (A, D) total and (B, E) phosphorylated PCSK9 secreted by radiolabeled cells were measured. Shown as a percentage of the total, the amount of phosphorylated PCSK9 decreased with increasing βE2 concdentation in HuH7 cells (C) but not in HepG2 cells (F) treated with 10 μM βE2. Data represent the mean ± SEM; $n = 3$ experiments, each performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0005$. Feno, fenofibrate.

we suggest a model wherein there is an additional level of regulation of PCSK9 function, and introduce a more dynamic role in its downstream regulation of LDLR (Fig. 7). We suggest that phosphorylated PCSK9 promotes degradation of LDLR, whereas nonphosphorylated PCSK9 is in an LDLR-protective state. The total level of LDLR is affected by the balance of these, whereby depletion of degradative PCSK9 would result in elevated LDLR levels, and, conversely, depletion of protective PCSK9 would result in reduced LDLR levels. Through βE2 treatment of HuH7 and HepG2 cells, and depletion media transfer experiments, we observed changes in levels of LDLR that support this theory.

Consistent with alternative cell and animal models, we found that βE2 induced a dose-dependent increase in LDLR protein levels in HuH7 cells (Fig. 1). The observed upregulation was inhibited by preincubation

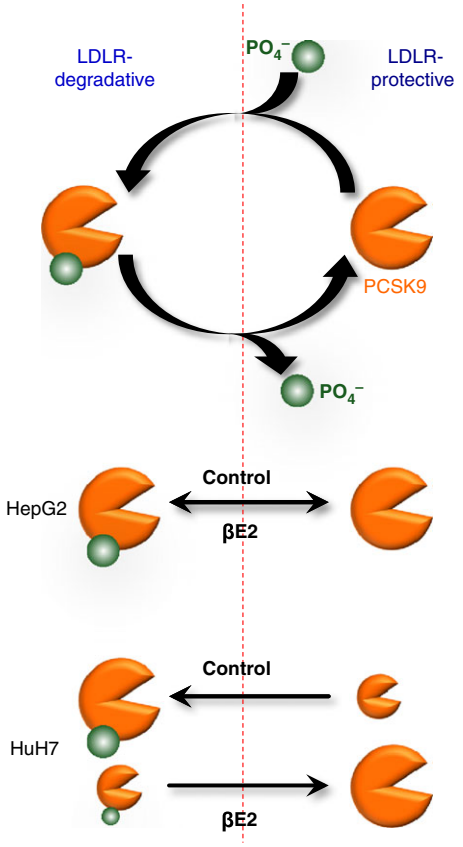

Fig. 7. Model for the functional state of PCSK9 in regulating LDLR levels. We propose a model wherein the state of PCSK9 is related to the activity on LDLR, acting both in promoting LDLR degradation (left side) and in having a protective function against LDLR degradation. The LDLR degradative activity would be related to elevated levels of phosphorylated PCSK9 (at one or more sites), whereas decreased phosphorylation of PCSK9 would be associated with protection of LDLR. The phosphorylation of PCSK9 may reduce interaction with a PCSK9 inhibitor, or directly or indirectly increase interaction with LDLR. Reduced levels of degradative (phosphorylated) PCSK9 would result in elevated LDLR levels, whereas depletion of protective (unphosphorylated) PCSK9 would result in lower LDLR levels. In HepG2 cells, the level of phosphorylation was not affected by βE2 treatment. In contrast, there was a significant reduction in the amount of phosphorylated PCSK9 secreted by HuH7 cells in response to βE2 treatment, thus promoting LDLR protection and the elevated levels observed *in vitro*.

of cells with fulvestrant, and thus was downstream of ER activation. Previous groups found similar results in HepG2 cells cotransfected with LDLR and ERα [7,27]; we found limited upregulation of LDLR at the protein level in HepG2 cells endogenously expressing LDLR and ERα (Fig. 5B).

By qPCR, we found that βE2 did induce transcriptional upregulation of *LDLR*; however, this occurred only at the highest dose tested (10 μM; Fig. 2), and was not sufficient to correlate with the elevated protein levels. As LDLR protein is negatively regulated by Idol and by PCSK9, we evaluated the expression of these regulators in βE2-treated HuH7 cells. Interestingly, βE2 increases Idol expression in cardiomyocytes [34]; however, there was no detectable change in Idol expression in HuH7 cells treated with βE2 (results not shown). Idol protein could not be detected with immunoblotting, despite evaluation with multiple commercially available antibodies, as has been previously observed for the endogenous expression of Idol in HuH7 cells [35,36]. Similarly, we did not observe any significant change in *PCSK9* transcription with increasing βE2 doses up to 10 μM, nor did we observe a change in the level of secreted PCSK9 (Fig. 2). Together, these findings are consistent with recent reports that overexpression of Idol in hepatic tissues can result in elevated PCSK9 expression [17]. However, the observed lack of a βE2-induced transcriptional response of *PCSK9* in HuH7 cells is in contrast to the previous results obtained with a rat model, which showed that *PCSK9* transcription was decreased by 50% in hepatocytes from rats treated with ethinylestradiol (a bioactive derivative of βE2) at 5 $mg \cdot kg^{-1} \cdot day^{-1}$ for 4 days [23]. The observed differences between the rat model and our current human hepatocyte model could be attributable to species differences. Notably, Srivastava *et al.* found consistent βE2-induced transcriptional regulation of *ldlr* between rat and mouse models, but different post-transcriptional mechanisms [37], suggesting that our observations may be species-specific. It would be interesting to compare the levels of circulating phosphorylated PCSK9 in different species to determine whether this could be a factor contributing to the differences. Regardless of the different findings for βE2-induced *PCSK9* transcription between human hepatic cells and rat hepatocytes, our results and those of Persson [23] both suggest that post-transcriptional regulation of LDLR occurs.

Although a change in the extracellular level of PCSK9 is associated with a change in LDLR level, Wooten *et al.* recently showed that elevated levels of PCSK9 are not the sole determinant of the interaction and subsequent degradation of LDLR in hepatic C3A cells [38]. Consistent with their findings, secreted PCSK9 levels did not change in the presence of βE2 in the current study, but there was a 'loss of effect' in terms of LDLR degradation. Interestingly, βE2 has also been shown to affect the activity of the high-density lipoprotein 1-associated enzyme paraoxonase 1 without altering the expression levels of this enzyme in HuH7 cells [39].

Removal of PCSK9 from βE2-treated versus vehicle-treated cell-conditioned media decreased recipient cell LDLR levels (Fig. 5A,D), indicating that extracellular PCSK9 (or an unidentified interacting protein) must be present for the upregulation of LDLR in response to βE2 to occur. Also, and in contrast to PCSK9-expressing HuH7 cells, shPCSK9 cells did not show an increase in LDLR levels following βE2 treatment (Fig. 4), indicating that the βE2-induced upregulation of LDLR is PCSK9-dependent. Interestingly, the depletion of PCSK9 from HepG2 control source media did not result in elevated levels of LDLR in HuH7 recipient cells; rather, there was a trend towards reduced LDLR levels (Fig, 6D). This suggests that the state of the PCSK9 that was removed from control HepG2 cell media was 'protective', and as such would push the balance towards LDLR degradation as compared with PCSK9-containing control media. To our knowledge, these results are the first to suggest that, under certain physiological conditions, the presence of PCSK9 stabilizes LDLR or extends its half-life.

Wooten *et al*. suggested that the functional state of PCSK9 may be dependent on an alternative, unidentified secreted protein [38]. We propose (Fig. 7) that βE2 causes a functional switch in the PCSK9–LDLR interaction, through a post-translational modification such as phosphorylation, and/or through upregulation of a PCSK9-inhibiting interactor that prevents its established function of escorting LDLR to the lysosome for degradation.

We previously showed that PCSK9 circulates as a phosphorylated protein [28]. Protein phosphorylation plays diverse functional roles within cells: it can activate and deactivate enzymes [40], affect signal transduction pathways [41], affect subcellular localization [42], alter protein stability [43,44], and modulate protein–protein interactions [45]. In 2008, we reported that the naturally occurring 'loss of function' PCSK9 variant R46L is hypophosphorylated at Ser47 in comparison with wild-type PCSK9, and that this correlates with increased cleavage of PCSK9′s propeptide following Ser47 [28]. Herein, we utilized differential radiolabeling of cells to show that there was a significant dose-dependent decrease in the amount of phosphorylated PCSK9 secreted in response to βE2 in HuH7 cells, but not in HepG2 cells (Fig. 6). The significant change in PCSK9 phosphorylation coincided with the increase in LDLR protein level without affecting transcription.

It is well recognized that the function of PCSK9 is highly dependent on the availability of specific structural sites within the protein. PCSK9 binds to LDLR through the catalytic domain [46], whereas the C-terminal domain of PCSK9 is required for promotion of LDLR degradation [47], and for interaction with the PCSK9 inhbitor annexin A2 [48–50]. We found no evidence of increased intracellular or extracellular annexin A2 levels in response to βE2 treatment of HuH7 cells (results not shown), suggesting the existence of an alternative inhibitory mechanism – potentially through a phosphorylation-dependent change in the PCSK9–annexin A2 interaction. Recently, it was shown that the PCSK9 prodomain binds to LDL particles through ApoB100, resulting in the inability of the protein to bind LDLR [51]. Additionally, antibodies directed against PCSK9 prevent the interaction of PCSK9 with LDLR, resulting in elevated levels of both LDLR and circulating PCSK9 [52]. Studies are ongoing to determine the mechanism by which βE2 inhibits the function of PCSK9, including evaluating the effect of PCSK9 phosphorylation on known protein interactions, and the effect of βE2 on PCSK9 and LDLR interactomes.

In humans, LDLC levels in premenopausal women are lower than in men, despite elevated PCSK9 levels [24,25]. It is tempting to speculate that – in light of our current findings – in premenopausal women the presence of the predominant female hormone βE2 induces a PCSK9 shift, whereby their circulating PCSK9 is not as 'effective' as their male counterpart's PCSK9 in terms of LDLR degradation. Future studies will further elucidate the hormonal regulation of the functional state of PCSK9.

Our results indicate that the observed increase in LDLR in response to BE2 treatment is dependent upon extracellular PCSK9, perhaps through a change in βE2-altered PCSK9 phosphorylation. This suggests a more dynamic role for PCSK9 than just escorting LDLR to the lysosome for degradation, but also that, in certain systems and conditions, such as in the HuH7 cell and under βE2 conditioning, PCSK9 or an as yet unknown PCSK9-interacting protein is necessary for LDLR upregulation at the protein level.

Experimental procedures

Cell culture

HuH7 cells (obtained from the Japanese Collection of Research Bioresources) and HepG2 cells (ATCC) were maintained at 37 °C and 5% CO_2 in DMEM (Invitrogen) supplemented with 10% qualified FBS (Invitrogen), 1 mM sodium pyruvate (Life Technologies, Burlington, ON, Canada), and gentamicin (28 $\mu g \cdot mL^{-1}$; Sigma-Aldrich, Oakville, ON, Canada). shRNA-mediated *PCSK9* knock-

down of HuH7 cells was performed by transfection of cells with the use of Lipofectamine 2000 (Invitrogen), as described by the manufacturer. Stable pools of shRNA-mediated *PCSK9* knockdown cells were selected and maintained by culture in 2 $\mu g \cdot mL^{-1}$ puromycin (Invitrogen). Two of Origene's (Rockville, MD, USA) shRNA constructs targeting human PCSK9 mRNA were used in combination for transfection (GCATACCTCACCAA GATCCTGCATGTCTT and CTTCTCTGCCAAAGATG TCATCAATGAGG), and a scrambled nontarget shRNA served as a control.

Cell stimulation

Experiments were performed in triplicate in six-well plates (6.5×10^5 cells per well). One day following plating, HuH7 or HepG2 cells were washed with PBS (pH 8.0; Invitrogen), and grown in serum-free, phenol red-free DMEM for 4–6 h before the addition of βE2 (Sigma-Aldrich) or vehicle (ethanol) in fresh serum-free DMEM at the doses indicated for an additional 48 h of growth, unless otherwise stated. When included in the experiment, the ER inhibitor fulvestrant (Sigma-Aldrich) or vehicle (DMSO; Sigma-Aldrich) was added in fresh serum-free DMEM 1 h prior to the addition of βE2. Alternatively, cells grown for 24 h in serum-free media were treated for an additional 24 h with 10 μM compactin (Mevastatin) as a positive control for LDLR upregulation. At the designated times, debris-free conditioned media were collected and stored at −20 °C for later ELISA. Cells were washed with PBS, and total cell lysates were extracted with RIPA buffer [radio-immunoprecipitation assay; 50 mM Tris (pH 7.6), 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) deoxycholate, 0.1% SDS] in the presence of a Complete Mini Protease Inhibitor Cocktail (Roche Diagnosics, Laval, PQ, Canada), as previously described [21]. Alternatively, after being washed with PBS, cells were incubated for 60 min at 4 °C with 0.5 $mg \cdot mL^{-1}$ EZ-Link Sulfo-NHS-LC Biotin (Thermo Scientific, Burlington, ON, Canada) in PBS (pH 8.5) to label cell surface proteins; the supernatant was discarded, and cells were washed with 50 mM Tris (pH 8.5) prior to cell lysate extraction with RIPA buffer. In cases where RNA was obtained, after media collection, PBS-washed cells were lysed for RNA isolation with 700 μL of RLT buffer (Qiagen Germantown, MD, USA) containing 1% 2-mercaptoethanol, with QIAshredder and RNeasy kits (Qiagen), according to the manufacturer's protocols. RNA was immediately stored at −80 °C.

Membrane-enriched protein fraction

Membrane-enriched protein fractions of HuH7 cells were isolated from lysates of EZ-Link Sulfo-NHS-LC Biotin-treated cells by immunoprecipitation with streptavidin agarose. Unbound fractions were collected ('membrane-depleted'), beads were washed three times with PBS, and membrane-enriched proteins were then eluted with sample-loading buffer at 70 °C for 10 min.

LDL uptake assay

HuH7 and HepG2 cells were grown as above in 96-well black clear-bottomed cell culture plates (15 000 and 30 000 cells per well, respectively) in phenol red-free media overnight at 37 °C. Following overnight incubation, cells were stimulated with 100 μL of 10 μM βE2 for 48 h in serum/phenol red-free media, with 10 μM compactin (Sigma Aldrich) for 24 h in serum/phenol red-free media containing βE2, or with ethanol as a vehicle control. Following stimulation, 10 μL of fluorescently labeled LDL (7 $mg \cdot mL^{-1}$; Biovision, Milpitas, CA, USA; LDL Uptake Assay Kit) was added to each well, and incubated at 37 °C for 60 min. Spent medium was removed, and wells were washed four times with 100 μL of Assay Buffer (Biovision; LDL Uptake Assay Kit). As a background control, 100 μL of serum/phenol red-free medium was added to unstimulated cells without fluorescently labeled LDL, and wells were washed as above. Fluorescence was measured at at excitation wavelength of 540 nm and an emission wavelength of 575 nm in a BIOTEK Synergy H1 Multi-Mode Plate Reader.

Immunoblot analysis

Total protein was quantified with the Bradford dye-binding method (Bio-Rad Protein Assay Kit, BioRad, Mississauga, ON, Canada). The levels of LDLR protein in equal amounts of total cell lysates and tissue lysates were measured by immunoblotting with standard laboratory protocols [21]. The commercial primary antibodies used were goat anti-human LDLR Ig (R&D Systems, Minneapolis, MN, USA; 1 : 500), rabbit anti-goat PCSK9 Ig (Circulex, Nagano, Japan; 1 : 1000), anti-(human transferrin receptor) Ig (Invitrogen, Burlington, ON, Canada; 1 : 2000), and mouse anti-actin Ig (Abcam, Cambridge, UK; 1 : 2000). Secondary antibodies were used at 1 : 5000 (Amersham, Mississauga, ON, Canada). Immunoblots were revealed by chemiluminescence with Western Lightning Plus (Perkin Elmer, MA, USA) on Progene film (Ultident Scientific, St. Laurent, QC, Canada). Densitometry was performed with IMAGEJ, and signals from total cell lysates or membrane-enriched fractions were normalized to the densitometry value for the corresponding actin or transferrin receptor signal, respectively. Values shown are relative to the normalized density of signals for control cells.

ELISA for secreted PCSK9

The levels of secreted human PCSK9 were quantified in duplicate from conditioned media with the CircuLex

human PCSK9 ELISA kit (CyClex, Nagano, Japan), according to the manufacturer's protocol; absorbance was measured at 450/550 nm with an Infinite F200 Pro Reader (Tecan, San Jose, CA, USA). This assay has an intra-assay coefficient of variation (CV) of 1.5–2.6% and an interassay CV of 2.9–7.1%.

RNA isolation and qPCR

Concentrations of RNA, isolated with RNeasy kits, were determined with the ND-1000 spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, DE, USA) and ND-1000 V3.5.2. Samples evaluated with agarose electrophoresis confirmed RNA integrity. DNase treatment was performed in a 10-μL reaction mixture for 15 min at 20 °C on 1 μg of sample RNA with 1 μL of DNase I Amp Grade (1 $U \cdot \mu L^{-1}$) in DNase I reaction buffer (Invitrogen), and stopped with 1 μL of 25 mM EDTA at 65 °C for 10 min.

cDNA was prepared by the use of random hexamer primers with SuperScript II Reverse Transcriptase followed by RNaseOUT treatment, in accordance with the manufacturer's protocol (Invitrogen). The lack of DNA contamination was confirmed with a pool of RNA in a reaction lacking reverse transcriptase.

Duplicate qPCR reactions were performed in a Stratagene Mx3000P PCR (Agilent Technologies, Santa Clara, CA, USA) with MxPro-Mx3000P on 2.5 μL of 10-fold-diluted cDNA in 12.5-μL reaction mixtures with SYBR QPCR low ROX Master Mix (Stratagene Products, Agilent Technologies) and 0.1 μM forward and reverse primers for PCSK9, LDLR, and β-actin (Table 1). Actin was used as a reference gene for consistency throughout experiments. Lack of contamination in primers, nucleic acid-free water and SYBR QPCR Master Mix was confirmed by reactions containing 2.5 μL of nucleic acid-free water instead of cDNA. The cycling conditions of the qPCR reactions were as follows: 94 °C for 10 min; 40 cycles of 95 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min. All qPCR reactions included a series of dilutions (five-fold, 10-fold, 25-fold, 125-fold, and 625-fold) of a pool of cDNA to assess the limit of detection and linear range of the qPCR reaction. As determined with MxPro-Mx3000P, the Cq of all samples showed positive signals that fell within the linear range of the standard curve. The intra-assay CV was determined to be 0.39–0.56%. All qPCR reactions included a no reverse transcriptase control and a no template control, none of which showed positive signals. Text reports were analyzed in Excel (Microsoft); the relative expression levels were calculated in accordance with Pfaffl [53].

βE2-treated PCSK9-depleted media transfer

Control or 10 μM βE2-treated conditioned medium from nontarget, shPCSK9 knockdown, HuH7 or HepG2 cells was collected, and cellular debris was removed by centrifugation at 2500 ***g***. In the case of HuH7 and HepG2 media transfer experiments, PCSK9 present in the source-conditioned media was removed by overnight immunoprecipitation with protein A-bound and protein G-bound agarose (Sigma Aldrich) at 4 °C in the presence of rabbit anti-human PCSK9 Ig [25]; immunoprecipitations performed in the absence of antibody served as media transfer PCSK9-present controls. Unbound fractions of source media were collected by centrifugation at 2500 ***g*** and filtration through 0.22-μm filters; aliquots were reserved for ELISA quantification of PCSK9. Filtered source media with PCSK9 or depleted of PCSK9 were transferred onto naive HuH7 cells and grown for 24 h prior to total cell lysate collection and immunoblotting analysis, as outlined above.

Measurement of secreted phosphoPCSK9

HuH7 cells were incubated with increasing concentrations of βE2 for 24 h prior to collection of conditioned media as described above. Alternatively, cells were incubated with 10 μM simvastatin (Sigma-Aldrich) or 200 μM fenofibrate (Sigma-Aldrich) prior to collection of conditioned media as described above. HuH7 cells were radiolabeled by growth in the presence of [^{35}S]Met/Cys or [^{32}P]orthophosphate, resulting in differential labeling of total proteins or phosphoproteins, as previously described [28]. Prior to radiolabeling, cells were incubated for 4 h in serum-free DMEM without sodium phosphate (Invitrogen) or for 45 min in Met/Cys-free DMEM (Invitrogen), and then incubated for 16 h in the same medium while βE2 and vehicle status was maintained in the presence of either 250 μCi of [^{32}P]orthophosphate or 250 μCi of [^{35}S]Met/Cys. Spent media were collected in the presence of a general protease inhibitor cocktail (Roche) and 200 μM sodium orthovanadate (a phosphatase inhibitor; Sigma-Aldrich), and centrifuged at

Table 1. Details of primers used for qPCR.

HUGO target	GenBank accession no.	Amplicon length	Exons: forward; reverse	Forward primer (5′- to 3′)	Reverse primer (5′- to 3′)
PCSK9	NM_174936	77	4; 5	TCACCGACTTCGAGAATGTG	GCCATGACTGTCACACTTGC
LDLR[a]	NM_000527	129	16/17; 17	GTGCTCCTCGTCTTCCTTTG	GCAAATGTGGACCTCATCCT
ACTB	NM_001101	117	4/5; 5	ACTCTTCCAGCCTTCCTTCC	AGCACTGTGTTGGCGTACAG

[a]The six splice variants of LDLR are all amplified by this primer.

13 000 ***g*** for 3 min to remove suspended cells and debris. PCSK9 immunoprecipitations were carried out in 1 × NaCl/Tris + 0.1% Tween-20 with anti-hPCSK9 IgG [25] (dilution 1 : 500) and 30 μL of protein A agarose (Sigma-Aldrich) overnight at 4 °C. Immunoprecipitates were washed four times with 1 mL of 1 × NaCl/Tris + 0.1% Tween-20, and fractionated through a 12% polyacrylamide gel. Following electrophoresis, gels were dried and visualized by phosphorimaging with a Typhoon Imager. Signals were quantified with IMAGEQUANT 5.2 software by use of the integer integration method when samples were compared within a lane, and with volume quantification when samples were compared between lanes, as recommended. Phosphorylated PCSK9 was quantified on the basis of the ratio of phosphorylated PCSK9 immunoprecipitated ([^{32}P]orthophosphate)/total PCSK9 immunoprecipitated ([^{35}S]Cys/Met), with control immunoprecipitates set as 1.

Statistical analysis

All data are presented as means ± standard errors of the mean (SEMs). Significant differences were assessed with Student's *t*-test or, where appropriate, with ANOVA followed Bonferonni's *post hoc* comparisons, with GRAPHPAD PRISM 5.0. *P*-values of < 0.05 were considered to be statistically significant.

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Author contributions

A. E. Starr and J. Mayne planned experiments, analyzed all data, and wrote the manuscript. A. E. Starr, V. Lemieux, J. Noad, J. I. Moore, T. Dewpura, A. Raymond and J. Mayne performed experiments. M. Chrétien, D. Figeys and J. Mayne provided essential funding and reagents. All authors contributed to manuscript editing.

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