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Effects of Proprotein Convertase Subtilisin Kexin type 9 modulation in human pancreatic beta cells function

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Non-Standard Abbreviations:

ABCA1, ATP binding cassette A1

AU, Arbitrary Unit

ECM, extracellular matrix

EI, equivalent islets number

FH, Familial Hypercholesterolemia

GSIS, Glucose Stimulated Insulin Secretion

HMGCR, 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase

HNF1α, Hepatocyte Nuclear Factor 1α

LDL, Low-Density Lipoprotein

LDL-C, LDL Cholesterol

LDLR, LDL Receptor

MFI, Mean Fluorescence Intensity

NOD, New onset diabetes

PCSK9, Proprotein Convertase Subtilisin Kexin Type 9

rPCSK9, recombinant PCSK9 D374Y

RMFI, Ratio of Mean Fluorescence Intensities

RT-PCR, Real Time-quantitative Polymerase-Chain Reaction

SREBP2, Sterol Response Element Binding Protein 2

T2D, Type 2 Diabetes

ABSTRACT (245 words)

Background and aims **-** Proprotein Convertase Subtilisin Kexin Type 9 (PCSK9) is an endogenous inhibitor of the LDL receptor (LDLR). Mendelian randomization studies suggest that PCSK9 deficiency increases diabetes risk, but the underlying mechanisms remain unknown. The aim of our study was to investigate whether PCSK9 or its inhibition may modulate beta cell function.

Methods **-** We assessed PCSK9 and insulin colocalization in human pancreatic sections by epifluorescent and confocal microscopy. We also investigated the expression and the function of PCSK9 in the human EndoC−βH1 beta cell line, by ELISA and flow cytometry, respectively. PCSK9 was inhibited with Alirocumab or siRNA. LDLR expression and LDL uptake were assessed by flow cytometry.

Results **-** PCSK9 was expressed and secreted from beta cells isolated from human pancreas as well as from EndoC−βH1 cells. PCSK9 secretion was enhanced by statin treatment. Recombinant PCSK9 decreased LDLR abundance at the surface of these cells, an effect abrogated by Alirocumab. Alirocumab as well as PCSK9 silencing increased LDLR expression at the surface of EndoC−βH1 cells. Neither exogenous PCSK9, nor Alirocumab, nor PCSK9 silencing significantly altered glucose-stimulated insulin secretion (GSIS) from these cells. High-low density lipoproteins (LDL) concentrations decreased GSIS, but the addition of PCSK9 or its inhibition did not modulate this phenomenon.

Conclusions **-** While PCSK9 regulates LDLR abundance in beta cells, inhibition of exogenous or endogenous PCSK9 does not appear to significantly impact insulin secretion. This is reassuring for the safety of PCSK9 inhibitors in terms of beta cell function.

Keywords – LDL receptor; Statins; PCSK9; PCSK9 inhibitors; Beta cell.

INTRODUCTION

Abnormalities in beta-cell function is closely associated with the risk of developing type 2 diabetes (T2D) (1,2). These defects comprise both a specific impairment of glucose-stimulated insulin secretion (GSIS) and a loss of beta cell mass (3). Changes in lipoproteins levels appear to impact beta cell function and survival, underpinning a role for lipoprotein particles in the pathogenesis of T2D (4). For instance, low-density lipoproteins (LDL) were shown to alter GSIS in pancreatic islets *ex vivo*, while high-density lipoproteins seem to exert protective effects (5).

In that respect, the prevalence of T2D is lower in patients with genetically reduced LDLR function (*i.e.* heterozygous familial hypercholesterolemia (HeFH)) than in unaffected relatives, and T2D prevalence is even lower in patients with the more severe HeFH phenotypes (*i.e.* those who carry *LDLR* negative mutations) (6). Conversely, statins that lower circulating LDL, primarily by enhancing *LDLR* gene expression, slightly but consistently increase the risk of new onset diabetes (NOD) in predisposed individuals (7,8).

Proprotein convertase subtilisin kexin type 9 (PCSK9) is a serine protease primarily but not exclusively expressed by the liver and secreted into the blood. PCSK9 binds to the LDLR and after endocytosis targets the LDLR for lysosomal degradation, preventing normal recycling of the receptor to the plasma membrane (9). PCSK9 may also inhibit LDLR abundance at the cell surface via an intracellular route, prior to secretion (10–12). Monoclonal antibodies targeting PCSK9 in the plasma constitute a novel class of LDL lowering agents preferentially used on top of statins to maximally up-regulate LDLR abundance at the cell surface (13,14), and thereby to further reduce the risk of cardiovascular events (15,16).

Activating the LDLR pathway in the liver is anti-atherogenic and clearly beneficial (9), but activating the same pathway in beta cells may be harmful to those cells, potentially by

impacting GSIS and increasing NOD, as it seems to be the case with statins. This hypothesis was recently underpinned by Mendelian randomization studies showing that loss-of-function genetic variants in *HMGCR* (the target of statins) and in *PCSK9* that activate the LDLR pathway and lower LDL-C levels are associated with an increased risk of NOD (17–19). Noteworthy, PCSK9 is expressed in human pancreatic islets and was initially found expressed in somatostatin producing pancreatic delta cells (20). In isolated human pancreatic islets however, *PCSK9* mRNA expression was found increased (+81±12%) in a sorted cells population enriched in beta cells but reduced (-75±5%) in a sorted cells population enriched in other islets cell types, compared with the entire population of human pancreatic islet cells (21,22). We have therefore investigated at the protein level the expression of PCSK9 in human pancreatic beta cells.

We showed that beta cells express and secrete PCSK9. In addition, we demonstrated that these cells express the LDLR at their plasma membrane and that LDLR abundance is duly regulated by exogenous as well as endogenous PCSK9. Finally, we showed that neither exogenous PCSK9 nor the PCSK9 inhibitor Alirocumab nor PCSK9 silencing with siRNAs significantly impact GSIS.

MATERIALS AND METHODS

Histological analyses - Human pancreas were obtained for research use from six brain-dead multi-organ donors under protocols approved by the Assistance Publique – Hopitaux de Paris (AP-HP) ethics committee, fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin sections mounted on glass slides (7µm thickness) were deparaffinized and rehydrated in a series of ethanol solutions of decreasing concentrations. Sections were washed with PBS and antigen retrieval performed in a 10mM Tris 1mM EDTA buffer (pH 9) for 10 minutes in a microwave oven (low intensity). Sections were placed in retrieval buffer at room temperature for 15 minutes and subsequently immersed in a 70% isopropanol solution containing 0.25% Sudan black B for 1h45 in the dark at room temperature, to quench pancreatic tissue auto-fluorescence (23). Sections were subsequently rinsed in isopropanol 70% and PBS, and blocked in PBS-5%BSA for 30 minutes. Antibodies were purchased from Abcam (Paris, France) or Invitrogen (Toulouse, France). Sections were incubated with primary antibodies (mouse anti human insulin/proinsulin ab8304 1:1000 dilution, rabbit anti human glucagon ab92517 1:50 dilution, rat anti human somatostatin ab30788 1:50 dilution, goat anti human PCSK9 C-terminal domain ab28770 1:50 dilution, rabbit anti human pro-PCSK9 N-terminal domain ab135647 1:25 dilution) in PBS-1%BSA for 1h30 at room temperature in a wet chamber. Sections were rinsed twice with PBS and incubated with the secondary antibodies (488 Alexa Fluor®-donkey anti-mouse A21292, 488 Alexa Fluor®-donkey anti-rabbit A21206, 488 Alexa Fluor®-goat anti-rat A11006, and 594 DyLight®-donkey anti-goat ab96937, 594 DyLight®-donkey anti-rabbit ab9692, respectively) diluted 1:200 in PBS-1%BSA for 1h30 at room temperature in a dark wet chamber, and counterstained with DAPI (1ng/mL). Sections were rinsed twice with PBS, mounted with coverslides in glycerol, and analyzed on an Eclipse 80i epifluorescence microscope and an A1 RSi confocal microscope

(Nikon, Champigny sur Marne, France). We systematically controlled the absence of cross-reactivity between all antibodies (**Supplemental Figure 1**). Colocalizations were determined using the Volocity 6.3 imaging software (Perkin Elmer, Milburn Hill Road NJ, USA).

Human Pancreatic beta cell line - All reagents were from Sigma-Aldrich (Saint Quentin Fallavier, France) unless stated otherwise. EndoC-βH1 cells (24) were purchased from Univercell Biosolutions (Toulouse, France). Cells were grown at 37°C on Extracellular Matrix (ECM) (100 µg/mL) and fibronectin (2µg/ml) coated flasks in DMEM containing 5.6mM glucose (Life Technologies, Saint Aubin, France), 2% BSA fraction V, 10mM nicotinamide (Merck Millipore, Fontenay Sous Bois, France), 50µM 2-mercaptoethanol, 5.5µg/mL transferrin, 6.7ng/mL sodium selenite, 100U/mL penicillin and 100µg/mL streptomycin (24). EndoC-βH3 cells (Univercell Biosolutions) were grown in the same medium supplemented with 10µg/ml puromycine (InvivoGen, Toulouse, France) (25).

LDLR expression - EndoC-βH1 cells were plated on ECM-fibonectin coated flat bottom 96-well plates (70,000 cells/well) in Optiβ1® medium (Univercell Biosolutions) containing 5.6mM glucose for 5 days at 37°C. Cells were starved by replacing the culture medium with Optiβ2® medium (Univercell Biosolutions) that contains only 2.8mM glucose with or without 10µg/mL mevastatin for 16h. Culture medium was replaced with fresh Optiβ2® medium with or without 10µg/mL mevastatin, and supplemented or not with 600ng/mL of recombinant PCSK9-D374Y (rPCSK9) (Circulex, Nagano, Japan) for 4h. In a subset of experiments, Alirocumab (Sanofi, Chilly-Mazarin, France), a fully human monoclonal antibody targeting PCSK9, was added concomitantly into the wells at a final concentration of 10µg/mL (13,14). In a subset of experiments, mevalonate (30µg/mL, Sigma) was added concomitantly with mevastatin into the wells. Cells were washed gently with PBS, lifted with Accutase®, re-

suspended in ice cold PBS, and washed twice in ice-cold PBS containing 1% BSA. Cells were incubated with an allophycocyanin-conjugated antibody against human LDLR (clone 472413) or an IgG1 (clone 11711) isotype control (R&D Systems) at 0.625µg/mL in PBS-1%BSA for 20 min at room temperature in the dark. Cells were then washed twice in ice-cold PBS-1% BSA and once in ice cold PBS before flow cytometry analysis on LSRII (Beckton Dickinson, Le Pont de Claix, FrancSe) or CYTOFLEX (Beckman Coulter, Villepinte, France) cytometers using the FlowJo software (Tree Star, Ashland OR, USA). Ratio between the mean fluorescence intensity (MFI) of cells incubated with the anti-LDLR antibody and the MFI of cells incubated with the isotype control was calculated to determine specific level of cell surface LDLR expression (RMFI), expressed in arbitrary units (AU) throughout.

LDL uptake - EndoC-βH1 cells were plated and treated as described above. LDL-BodipyFL (Life Technologies, Saint Aubin, France) was added to the medium at a final concentration of 10µg/mL for the last 3h of the incubation period (*i.e.* with or without rPCSK9). Cells were washed twice in ice-cold PBS-1% BSA, once in ice-cold PBS and re-suspended in ice-cold PBS supplemented with 0.2% trypan blue to quench cell surface-bound fluorescent LDL prior to flow cytometry analysis (13). Ratio between the MFI of cells incubated with LDL-bodipyFL and the MFI of cells incubated without LDL-BodipyFL (background fluorescence) was calculated to determine specific level of internalized LDL (RMFI), expressed in arbitrary units (AU) throughout. For confocal microscopy analyses, cells were seeded on ECM-fibonectin coated 8-wells Millicell EZ slide (Merck-Millipore, Fontenay Sous Bois, France) and treated as described above. Cells were washed twice in ice-cold PBS-1%BSA and once in PBS before fixation in PBS containing 4 % paraformaldehyde for 15min at room temperature. After an additional wash in PBS, slides were mounted with coverslides in Prolong anti-fade

reagent containing DAPI (Life Technologies) and visualized on a confocal A1 RSi microscope (Nikon, Melville, USA) (13).

Isolated human islets and PCSK9 secretion assay - Human pancreas were harvested from two brain-dead adult human donors (one man, 39 years, 24.3 BMI and one woman, 52 years, 19.8 BMI) in the context of the traceability requirements for the clinical islet transplantation program from the University hospital center of Lille, France (clinicaltrials.gov, NCT01123187, NCT00446264, NCT01148680). Isolation and islet culture were performed as described (26) and experiments were carried out on human islets isolated from both donors with 96.5% and 94.5 % viability and 80% and 90% purity (endocrine versus exocrine tissue), respectively. Purified human islets were cultured in CMRL-1066 medium (Life Technologies) with 5mM glucose, 100U/ml penicillin, 100µg/ml streptomycin and 0.625% Human Serum Albumin (Vialbex, LFB, Courtaboeuf, France) in a humidified atmosphere with 5% CO2 at 37°C. After 24 hours, islets were washed in RPMI medium (Life Technologies) and distributed in 24 wells plate at a density of 200 Equivalent Islets number (EI) per well. Islets were then incubated in RPMI medium containing 2.8mM glucose, 0.5% Fetal Bovine Serum with or without mevastatin (5µg/mL). After 20 hours incubation, culture media were collected and centrifuged to get rid of cell debris and PCSK9 concentrations were measured by ELISA using the PCSK9 Quantikine ELISA kit (R&D Systems). EndoC-βH cells were plated and treated as described above in the absence or presence of mevastatin, simvastatin, or pravastatin (10µg/mL). The supernatants were collected and centrifuged to get rid of cell debris and PCSK9 concentrations were measured by ELISA.

Glucose-stimulated insulin secretion (GSIS) - EndoC-βH1 cells were plated on ECM-fibonectin coated flat bottom 12-well plates (375,000 cells/well) in Optiβ1® medium for 5

days at 37°C. Culture medium was replaced with Optiβ2® medium with 0 or 10µg/mL mevastatin, and 0 or 600ng/mL rPCSK9 for 24h at 37°C. In a subset of experiments, Alirocumab or human LDL isolated from human plasma by sequential ultracentrifugation ($1.006 < d < 1.063$ g/mL) at 40,000g and dialyzed extensively against PBS before use, were added concomitantly into the wells at final concentrations of 10µg/mL and 250mg/dL, respectively. GSIS was performed as follows: i) cells were washed with βKREBS® buffer (Univercell Biosolutions) supplemented with 0.1% BSA and subsequently incubated in βKREBS® buffer-0.1% BSA without glucose for 40min at 37°C. ii) Supernatants were collected on ice and replaced with βKREBS® buffer-0.1% BSA supplemented with 20mM D-glucose for an additional 40min at 37°C. iii) supernatants were collected on ice and cells were lysed on ice in lysis buffer containing Tris 0.02M, Triton X100 1%, Glycerol 10%, 0.137M NaCl, 2mM EGTA and anti-protease (Roche diagnostics) for 10 min. Insulin contents in supernatants collected sequentially and in cell lysates, were determined by ELISA (Mercodia, Uppsala, Sweden). GSIS was assessed as the ratio of insulin content in the second supernatant (post-glucose stimulation) to insulin content in the first supernatant (pre-glucose stimulation). The initial insulin content per well was the sum of the insulin contents measured in cell extracts and both supernatants. In a subset of experiment, GSIS was performed with EndoC-BH1 silenced for PCSK9 (see paragraph below). Optimal concentrations of glucose to assess GSIS in EndoC-βH1 cells was determined by measuring insulin secretion following 40 min treatment with increasing concentrations of glucose (*i.e.* 0, 2.8, 5, 15, 20 and 25 mM), as described above (**Supplemental Figure 2**). Of note, we did not observe any change in cell viability, as assessed using the lactate deshydrogenase cytotoxicity detection kit ab102526 (Abcam), at any of the glucose concentrations tested or in the absence of glucose for 40 min (*data not shown*).

Statistical analyses – All results in the text and figures are presented as mean ± SEM. All statistical comparisons were performed using a Mann-Whitney test with GraphPad Prism 6.01 (La Jolla, CA, USA). $p<0.05$ indicates statistical significance.

RESULTS

Human beta cells express, synthesize and secrete PCSK9 - To demonstrate that beta cells are the islet cell type expressing PCSK9, we assessed PCSK9 protein expression in pancreatic sections from six donors by epi-fluorescent and confocal microscopy. Pancreatic sections were immuno-stained for human PCSK9 as well as for insulin, glucagon, or somatostatin. We first observed by epi-fluorescence that PCSK9 expression was restricted to pancreatic islets (**Figure 1 A-O**). We also observed that PCSK9 appeared to localize in insulin-positive cells, but definitely not in glucagon-positive or somatostatin-positive cells, indicating that PCSK9 protein expression is likely restricted to beta cells in human pancreatic islets. Using a specific antibody targeting the autocatalytic cleavage site of PCSK9, we verified that pro-PCSK9, the zymogen of PCSK9, was also expressed in insulin-positive cells (**Figure 1 P-T**). The co-localization between PCSK9 and insulin as well as the total absence of co-localization between PCSK9 and glucagon or somatostatin were fully ascertained using a high-resolution confocal microscope (**Figure 2 A-L**). Refined imaging analyses showed subcellular colocalization of insulin and PCSK9, indicating that both proteins colocalize in the same organelles (**Figure 2 M-Q**), although not exclusively. Similar observations were made independently in all pancreas sections from each of our six donors.

We next verified whether human beta cells could secrete PCSK9. We therefore assessed PCSK9 protein secretion in the supernatant of a unique human clonal beta cell line, the EndoC-βH1 cells. These cells secreted substantial amounts of PCSK9 (**Figure 3**). Since among all currently prescribed lipid lowering treatments, statins are the most potent at up-regulating PCSK9 in hepatocytes, (27,28), we next measured PCSK9 secretion from EndoC-βH1 cells following 20 hours treatment with statins. PCSK9 secretion was significantly increased following incubation with mevastatin, simvastatin and pravastatin (**Figure 3A**).

Since silencing of the *SV40* immortalization transgene in EndoC-βH1 cells leads to a phenotype close to that of β-cells in an adult pancreas (*i.e.* elevated intracellular insulin content and reduced proliferative capacity) (29), we ascertained whether PCSK9 secretion from these cells was maintained at comparable levels under these conditions. We silenced the *SV40* immortalization transgene of EndoC-βH1 cells (**Supplemental Figure 3A**) and verified that PCSK9 secretion from these cells remained steady (**Supplemental Figure 3C**). Of note, following *SV40* silencing, the intracellular insulin content of EndoC-βH1 increased by 90% indicative of an enhancement of the β-cell specific features mentioned above (**Supplemental Figure 3B**) (30). Mevastatin treatment increased PCSK9 secretion by 92% in silenced EndoC-βH1 cells (*data not shown*). Results obtained in EndoC-βH1 cells were confirmed in another line of human clonal beta cells, the EndoC-βH3, derived from a distinct donor (25). EndoC-βH3 secreted similar amounts of PCSK9 than EndoC-βH1 under basal conditions, and PCSK9 secretion also increased upon statin treatment in this cell line (*data not shown*). In addition, we showed that PCSK9 was secreted from pancreatic islets isolated from two human donors, an effect significantly increased upon treatment of the islets with mevastatin (**Figure 3B**). Taken together, these results demonstrate that human beta cells synthesize and secrete PCSK9 and that PCSK9 expression is increased by statin treatment in beta cells.

LDLR expression is regulated both by exogenous and endogenous PCSK9 in human beta cells - To determine whether PCSK9 alone or on top of statins modulates LDLR expression in human beta cells, we next assessed cell-surface LDLR expression in EndoC-βH1 cells treated with or without mevastatin, recombinant PCSK9 gain-of-function D374Y (rPCSK9), and/or Alirocumab (**Figure 4A**) by flow cytometry. Under basal culture conditions, the abundance of the LDLR at the plasma membrane of EndoC-βH1 was found at RMFI levels of 40.3±4.1 AU. Mevastatin treatment significantly increased LDLR cell surface expression (+104%). In

contrast, exogenous rPCSK9 alone or on top of mevastatin, significantly reduced the abundance of the LDLR at the surface of these cells by 75% and 66%, respectively (**Figure 4A**). We also tested the PCSK9 inhibitor Alirocumab under these experimental conditions. Alirocumab alone did not significantly alter the levels of LDLR expressed at the surface of EndoC-βH1 cells. However, on top of mevastatin (*i.e.* when the secretion of PCSK9 endogenously produced by these cells is increased), Alirocumab significantly increased LDLR cell surface expression by 20%. Alirocumab restored the levels of LDLR expression at the surface of EndoC-βH1 cells treated with rPCSK9 in the absence as well as in the presence of mevastatin. This was further validated by the concomitant use of mevalonic acid which reverts the effects of mevastatin on LDLR expression in this particular cell type as well (**Figure 4A**). Since PCSK9 has been shown in murine macrophages to also inhibit the expression of ABCA1 transporter (31), a key player in high-density lipoprotein metabolism, we ascertained under the experimental conditions described above the expression of ABCA1 in EndoC-βH1 cells. We did not observe any significant effect of mevastatin, rPCSK9 or Alirocumab on the level of ABCA1 expression in human clonal beta cells. A representative western blot is shown in **Supplemental Figure 4**.

We next assessed the cellular uptake of fluorescent LDL in EndoC-βH1cells by flow cytometry, using validated protocols (32) (**Figure 4B**). LDL uptake was found at RMFI levels of 221±5 AU under basal culture conditions. In line with the variations in LDLR cell surface expression described above, mevastatin increased fluorescent LDL uptake by 37% in these cells, whereas rPCSK9 alone or on top of mevastatin significantly reduced fluorescent LDL uptake by 83% and 52% respectively (**Figure 4B**). Variations in fluorescent LDL uptake upon these experimental conditions were visualized by confocal microscopy and paralleled the patterns of expression of the LDLR at the surface of EndoC-βH1cells (**Figure 4C**).

To specifically test the effect of endogenous PCSK9 on LDLR expression, we next silenced *PCSK9* in EndoC-βH1 using a validated siRNA (**Supplemental Methods & Results, Supplemental Figure 5).** We assessed PCSK9 secretion as well as cell surface LDLR expression as described above (**Figure 4D-E**). Under these conditions, PCSK9 silencing reduced the secretion of PCSK9 in the culture medium (-47%), which paralleled a significant increase in cell surface LDLR abundance (+28%). These combined results indicate that LDLR expression is modulated by statins and by both endogenous and exogenous PCSK9 in human beta cells and that these effects are reversed upon PCSK9 inhibition.

PCSK9 does not significantly alter GSIS - To establish whether PCSK9 alters insulin secretion from human beta cells, we undertook a series of insulin secretions measurements (GSIS) using the EndoC-βH1cell line grown in a culture medium containing or not mevastatin, rPCSK9, and/or Alirocumab **(Figure 5)**. We found that neither rPCSK9 nor mevastatin significantly modulated GSIS **(Figure 5A)** and neither did we observe any significant change in intra-cellular insulin contents **(Figure 5B)**. Likewise, the PCSK9 inhibitor Alirocumab did not significantly alter GSIS from EndoC-βH1 cells in each of the experimental conditions tested (**Figure 5C**). We next assessed the impact of *PCSK9* silencing on GSIS from EndoC-βH1 cells. Whereas *PCSK9* silencing increased LDLR cell surface expression, it did not significantly alter GSIS from these cells (**Figure 5D**). We also investigated the effects of rPCSK9 with or without mevastatin on EndoC-βH1 cells exposed to high concentrations of purified human LDL, that have been reported to reduce GSIS (5) (**Figure 5E-F).** The exposure of beta cells to LDL led to a 49% decrease in GSIS without altering intra-cellular insulin contents. PCSK9 alone or on top of mevastatin did not significantly modulate GSIS from EndoC-βH1 cells exposed to LDL. Taken together these results indicate that PSCK9 and Alirocumab do not alter GSIS from human beta cells.

DISCUSSION

In an attempt to elucidate the role of PCSK9 in the endocrine pancreas, we have shown that PCSK9 is expressed, synthesized and secreted from human beta cells. We have also established that exogenous PCSK9 modulates the expression of the LDLR at the plasma membrane of the EndoC-βH1 human pancreatic beta cell line. This effect was abolished in the presence of the PCSK9 inhibitor Alirocumab. Reassuringly, neither statins nor PCSK9 nor Alirocumab significantly altered the ability of human beta cells to secrete insulin in response to glucose.

It is well established that the LDLR is expressed in the endocrine pancreas, in particular in beta cells (33–35). In the present study, we demonstrate that PCSK9 is expressed in insulin producing human beta cells, in line with previous studies showing substantial PCSK9 expression in rodent beta cell lines (36,37) and in human pancreatic islets (20). We also showed that neither alpha cells nor delta cells significantly express PCSK9, discrepant with previous reports showing PCSK9 expression in delta cells by immunohistochemistry (20,38). We do not have a clear explanation for this discrepancy beyond technicalities (different antibodies were used), but recent mRNA data clearly indicates a specific endogenous expression of *PCSK9* in human beta cells (21,22), furthered by our observation of pro-PCSK9 in this cell type. The up-regulation of PCSK9 secretion by statins in two human EndoC-βH cell lines, similar to what occurs in hepatocytes (27), underlines the physiological pattern of PCSK9 expression in this particular cell type. Yet the relevance of the colocalization between PCSK9 and insulin/proinsulin in beta cells subcellular compartments remains to be established. To date, only one convertase (PCSK1) is known to directly interact with insulin, as it promotes proinsulin cleavage into insulin and C-peptide (39). Noteworthy, *PCSK9* gene expression is induced by the transcription factor HNF1α (hepatocyte nuclear factor 1α), a

major activator of the insulin gene (40). An interesting avenue would be to determine whether *PCSK9* gene expression in β-cells is primarily modulated by HNF1α or via the canonical SREBP2 (sterol response element binding protein 2) pathway.

It is also well established that LDLR abundance at the surface of many cell types is positively modulated by statins and negatively modulated by circulating PCSK9 primarily via the endo-lysosomal degradation route (9). Here we showed in human EndoC-βH1 that LDLR cell surface expression is increased by statin treatment and reduced by exogenous rPCSK9, as it is the case in almost every single cell type or tissue except the adrenals (41–43). The effect of exogenous rPCSK9 on LDLR expression was abolished by the PCSK9 inhibitor Alirocumab. Our results extend the observations made in previous studies showing that PCSK9 deficiency is associated with higher LDLR expression in whole mouse pancreas and isolated islets, and that the addition of exogenous PCSK9 reduces LDLR expression in human isolated islets (20,37). Noteworthy, we did not observe any effect of Alirocumab alone on LDLR expression under basal culture conditions, an observation no longer valid when beta cells were stimulated with mevastatin. It is likely that the amount of PCSK9 endogenously produced and secreted from human clonal beta cells is not large enough to significantly modulate LDLR cell surface expression under basal culture conditions but not after stimulation with mevastatin. We also showed that the addition of exogenous rPCSK9, mevastatin, or Alirocumab did not significantly alter GSIS, even in lipotoxic culture conditions (*i.e.* in presence of high LDL concentrations). EndoC-βH1 cells are perfectly suited to study GSIS, as no other human beta pancreatic cell line secretes insulin in a dose dependent manner in response to glucose (44). In agreement with these results, GSIS from wild type or PCSK9 knockout mice pancreatic islets were found similar in a previous study (20). The absence of effect of PCSK9 inhibition with Alirocumab on GSIS in our cellular model is in line with the fact that neither Evolocumab (15) nor Alirocumab (16) have shown any sign of increased incident cases of diabetes in

clinical trials so far. In agreement with these observations, we recently reported that plasma PCSK9 concentrations do not predict the risk of T2D in subjects with prediabetes (45).

Based on the results of the present study, we can cautiously conclude that PCSK9 is physiologically expressed in human beta cells and that circulating PCSK9 (and hence PCSK9 inhibition with mAbs) has the ability to regulate LDLR abundance at the surface of these cells without directly impacting their ability to secrete insulin in response to glucose. Taken together, the present results are reassuring regarding the safety of PCSK9 inhibitors in terms of beta-cell function (46,47). However, regarding the potential link between PCSK9 and glucose homeostasis raised by Mendelian randomization studies, it is conceivable that the effect of PCSK9-deficiency on glucose homeostasis may not be restricted to pancreatic islets or insulin secretion. For instance, plasma PCSK9 concentrations have been found to be positively associated with markers of insulin resistance such as HOMA-IR or hepatic glucose production assessed during hyperinsulinemic-euglycemic clamps (48,49). Additional studies are therefore warranted to decipher the precise role of PCSK9 in diabetes risk.

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FIGURE LEGENDS

FIGURE 1 – Total PCSK9 and pro-PCSK9 expression in human pancreas is restricted to Langerhans Islets cells - Epifluorescent microscopic analyses of human pancreatic sections immuno-stained for human PCSK9 C-terminal domain **(A-O)** or N-terminal domain **(P-T)**, insulin **(A-E and P-T)**, glucagon **(F-J)**, or somatostatin **(K-O)**. Entire pancreatic sections are shown in panels (**A**), **(F)**, (**K**) and (**P**). The 25µm scale is for all panels except (**A**), (**F**), (**K**) and (**P**).

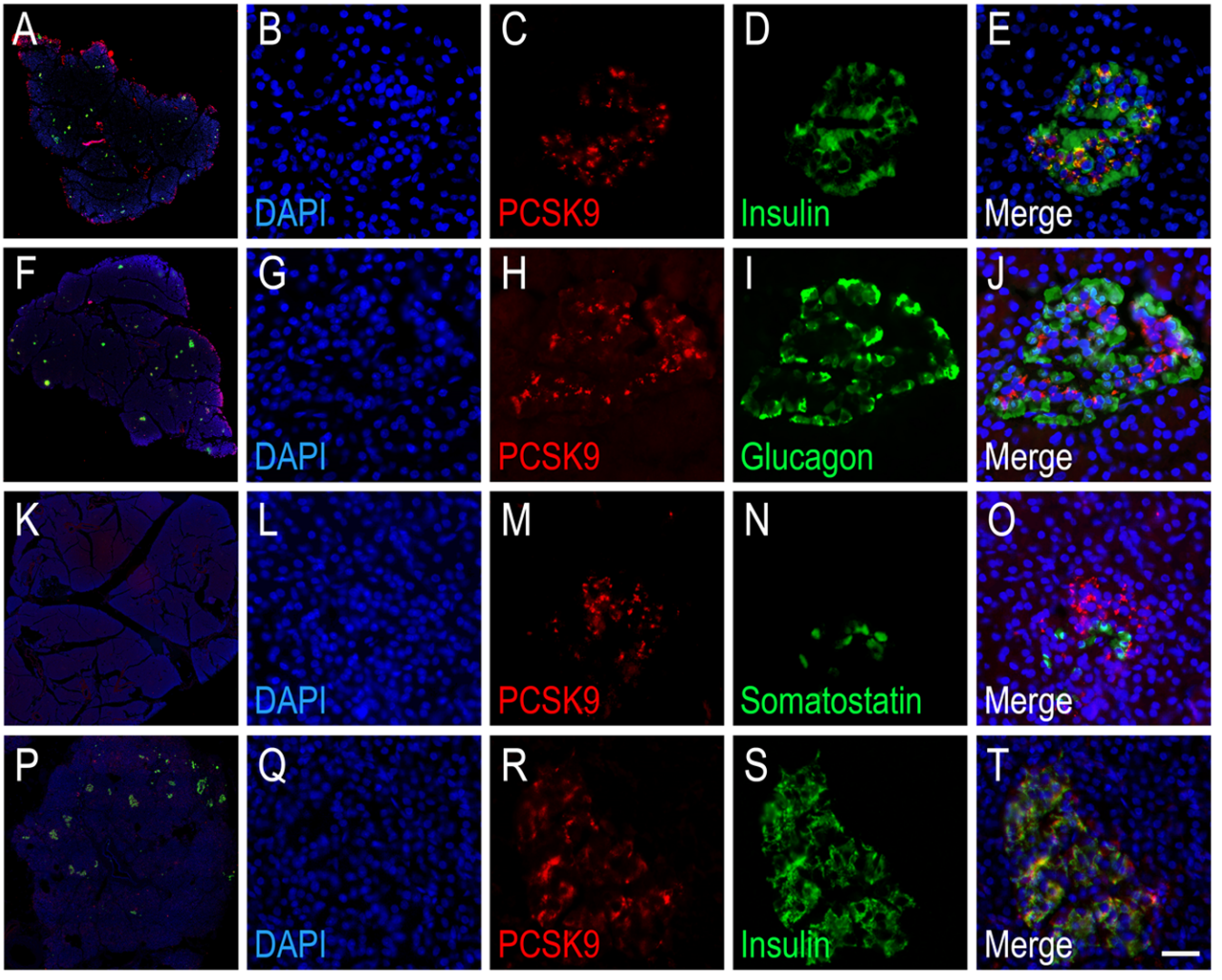
FIGURE 2 - PCSK9 expression in human pancreatic islets is restricted to beta cells and colocalizes with insulin - Confocal microscopic analyses of human pancreatic sections immuno-stained for human PCSK9, insulin **(A-D)**, glucagon **(E-H)**, or somatostatin **(I-L)**. Accurate colocalization patterns between insulin and PCSK9 within islets beta cells are shown in panels (**M-Q)** and calculated as the product of the difference from the mean (PDM) with Volocity **(P)**. Scales: 15µm **(panels A-L)** and 10µm **(panels M-Q)**.

FIGURE 3. PCSK9 is secreted from human clonal beta cells and from isolated human pancreatic islets - (Panel A) EndoC-βH1 cells were treated for 20 hours with or without mevastatin, pravastatin or simvastatin (10µg/mL) and PCSK9 secretion was measured in the culture medium collected during the last 4 hours of the incubation period. Results are expressed as mean±SEM per10^6 seeded cells (n=5 independent experiments performed in duplicates for mevastatin and in simplicate for pravastatin and simvastatin). **(Panel B)** Human pancreatic islets isolated from two donors were treated with or without 5µg/mL mevastatin for 20 hours. Mean±SEM of PCSK9 secretion from five independent experiments conducted in 200 seeded Equivalent Islets (EI) each within the 20 hours incubation period are shown. * $p<0.05$ and ** $p<0.01$ vs. no statin.

FIGURE 4 - Mevastatin increases whereas PCSK9 reduces LDLR cell surface expression in human clonal beta cells. EndoC-βH1 cells were treated for 16 hours with or without mevastatin and subsequently for 4 hours with or without mevastatin, recombinant PCSK9-D374Y (rPCSK9), mevalonate, and/or Alirocumab. **(Panel A)** Cell surface LDLR expression was measured by flow cytometry. In a subset of culture wells, fluorescent LDL was added to the medium for the last 3 hours of the incubation period. LDL uptake was measured by flow cytometry **(panel B)** and visualized by confocal microscopy **(panel C,** a

control, b PCSK9, c mevastatin, d PCSK9 + mevastatin). EndoC-βH1 cells were transfected with a siRNA targeting PCSK9 or a non-targeting control siRNA (siRNA control). On day 3 post-transfection, PCSK9 secretion within 20 hours was measured by ELISA **(panel D),** and LDLR cell surface expression was assessed by flow cytometry **(panel E)**. Results are expressed as mean±SEM, **p*<0.05; ***p*<0.01; ****p*<0.001; *****p*<0.0001.

FIGURE 5 - PCSK9 does not significantly alter GSIS in human clonal beta cells. EndoC-βH1 cells incubated for 24h in Optiβ2® medium containing 2.8mM glucose were treated with or without mevastatin and rPCSK9. GSIS **(panel A)** and initial insulin contents **(panel B)** were assessed (*n=10*). In a subset of experiments Alirocumab was tested **(panel C)** (*n=4*). EndoC-βH1 cells were transfected with a siRNA targeting PCSK9 or a non-targeting control siRNA (siRNA control). On day 4 post-transfection, GSIS was assessed **(panel D)** (*n=4*). Alternatively, EndoC-βH1 cells incubated for 24h in Optiβ2® medium containing 2.8mM glucose were treated with or without mevastatin, rPCSK9 and supplemented with 0 or 250 mg/dL of purified human LDL. GSIS **(panel E)** and initial insulin contents **(panel F)** were assessed (*n=7*). *p<0.05 vs. no LDL; ## p<0.01 vs. LDL alone.


A
B
DAPI
C
PCSK9
D
Insulin
E
Merge
F
G
DAPI
H
PCSK9
I
Glucagon
J
Merge
K
L
DAPI
M
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N
Somatostatin
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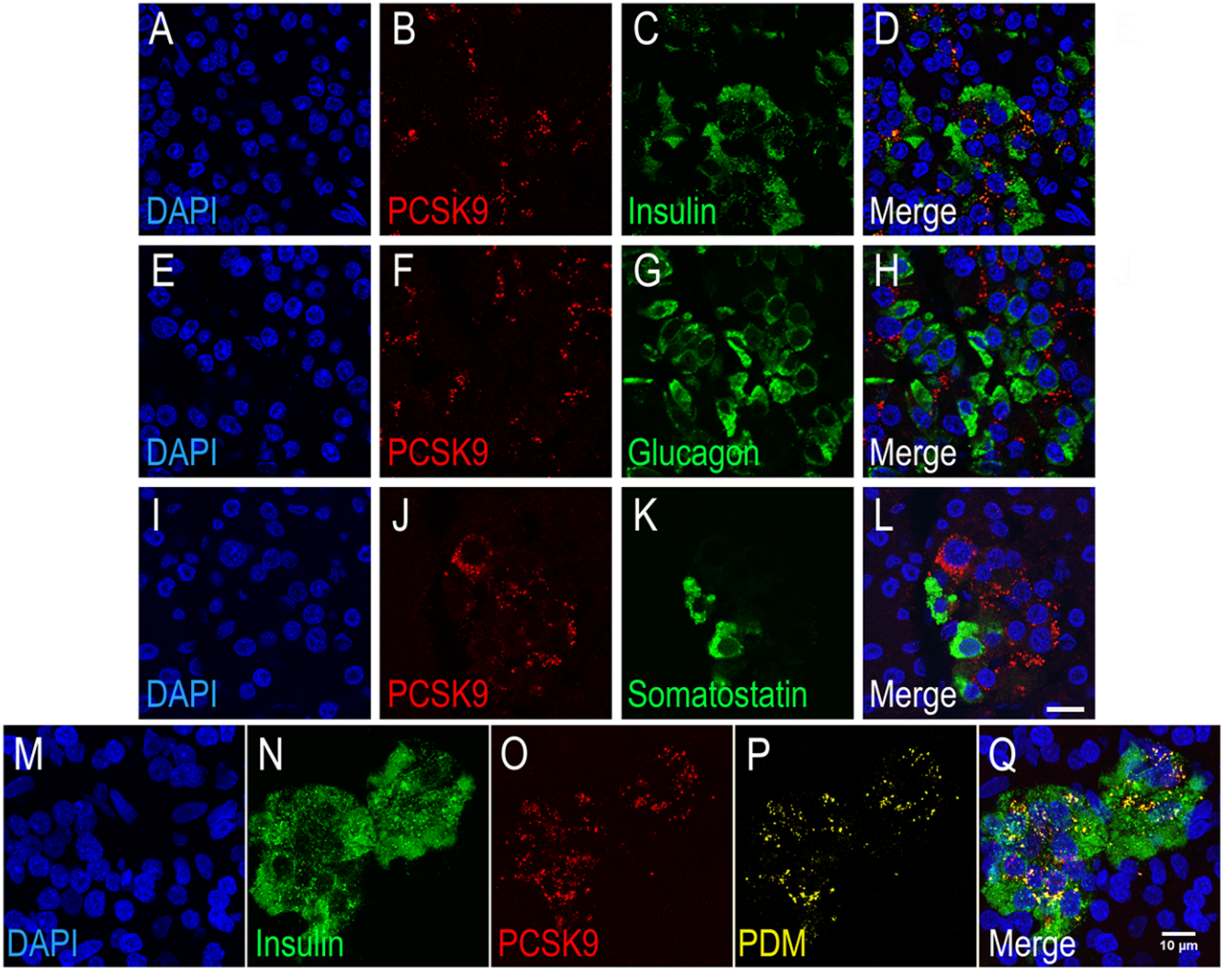
P
Q
DAPI
R
PCSK9
S
Insulin
T
Merge


A
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Merge
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M
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N
Insulin
O
PCSK9
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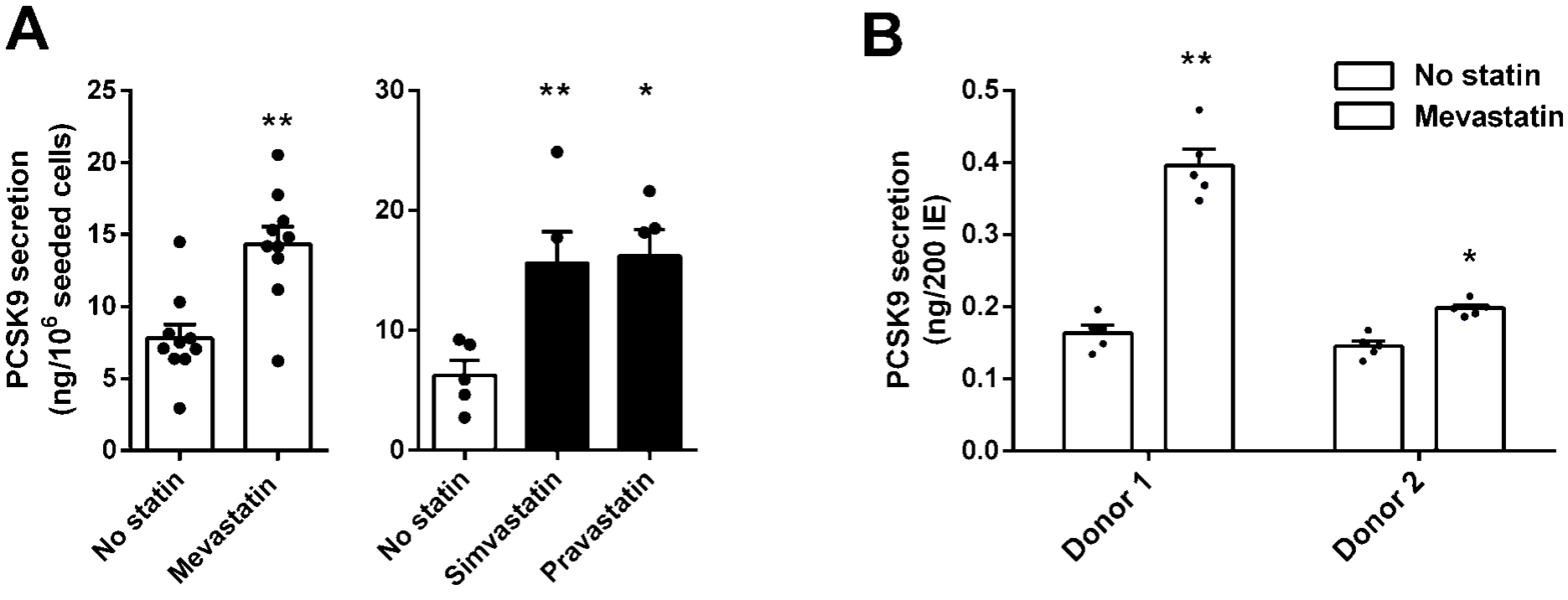
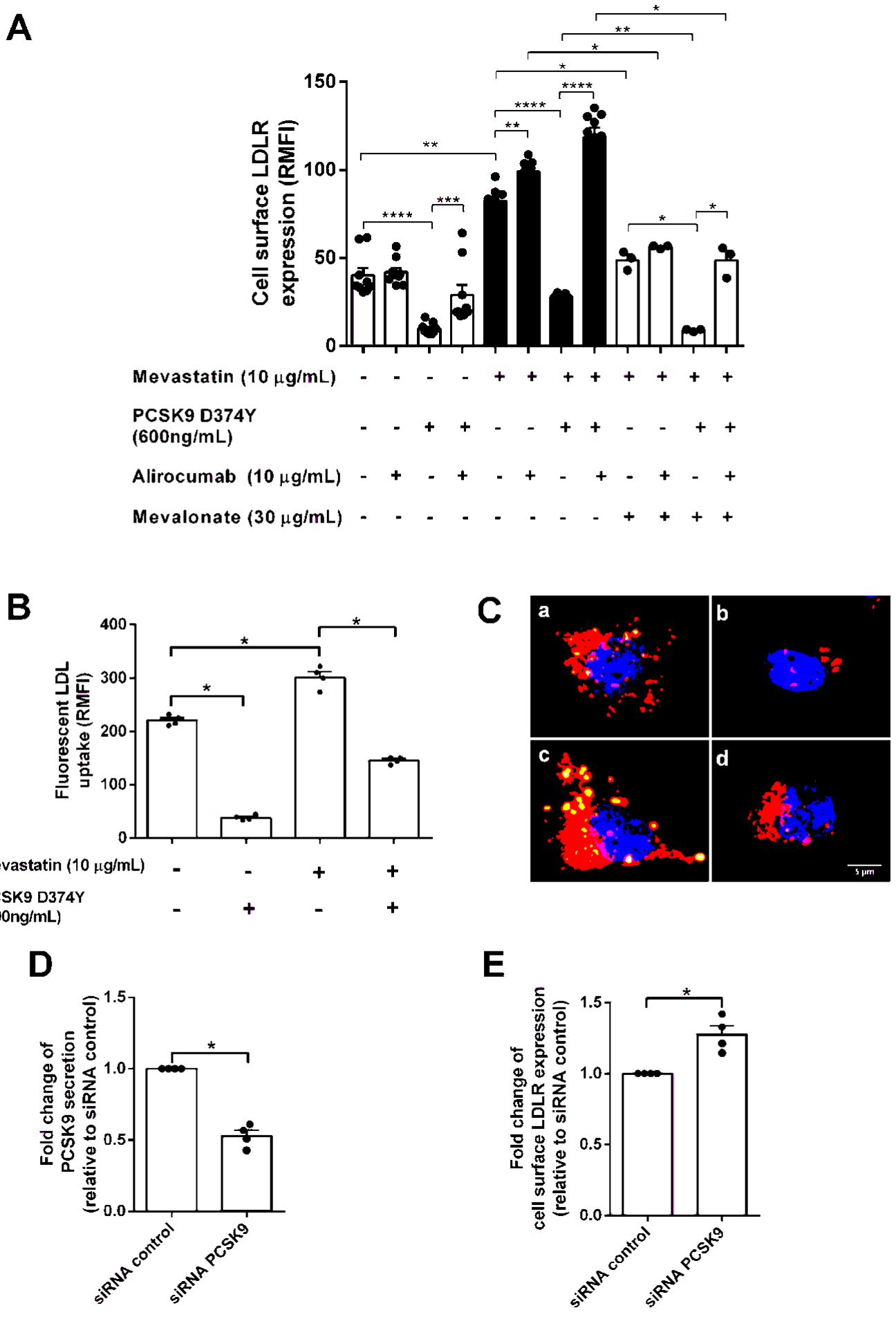
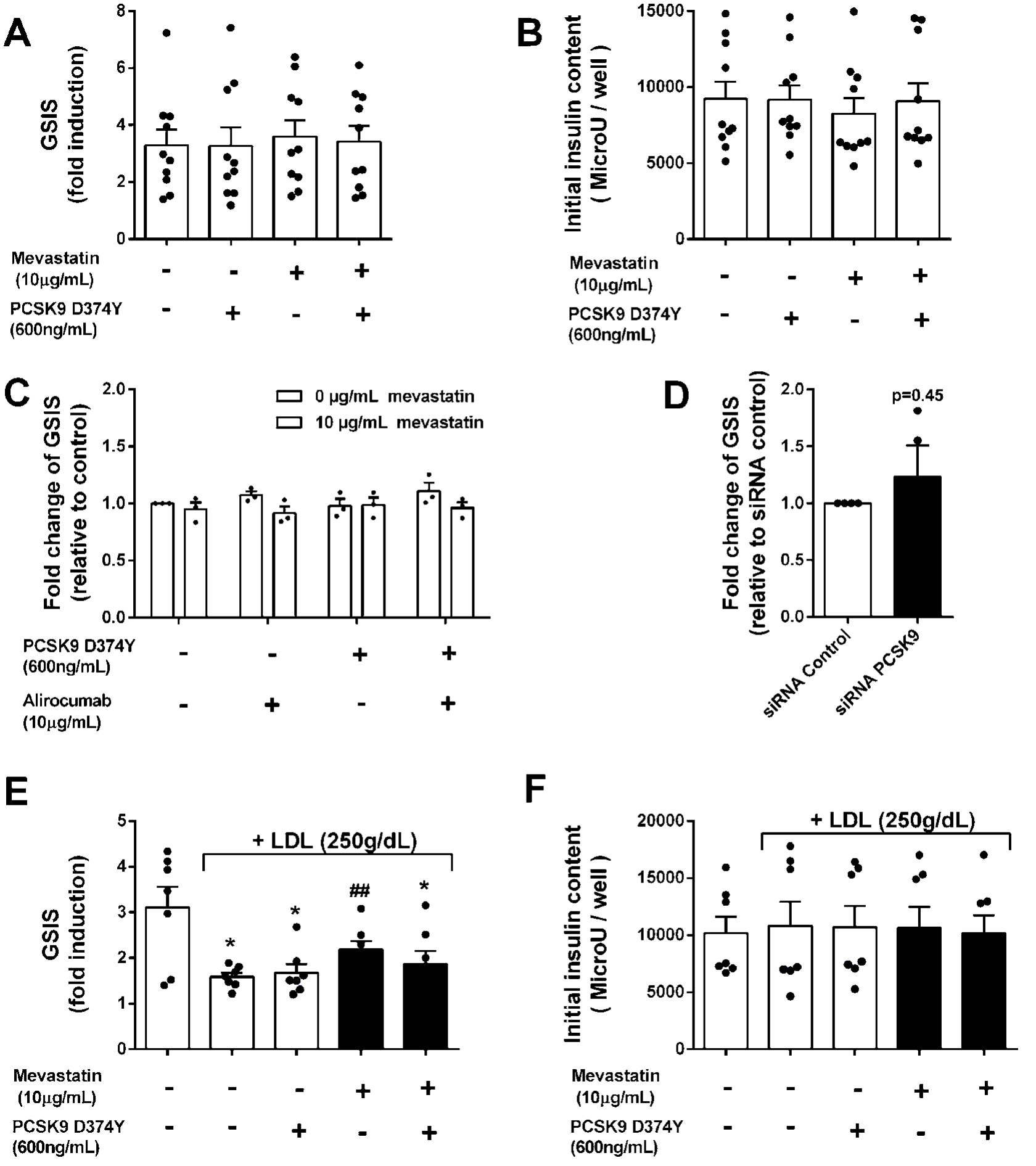
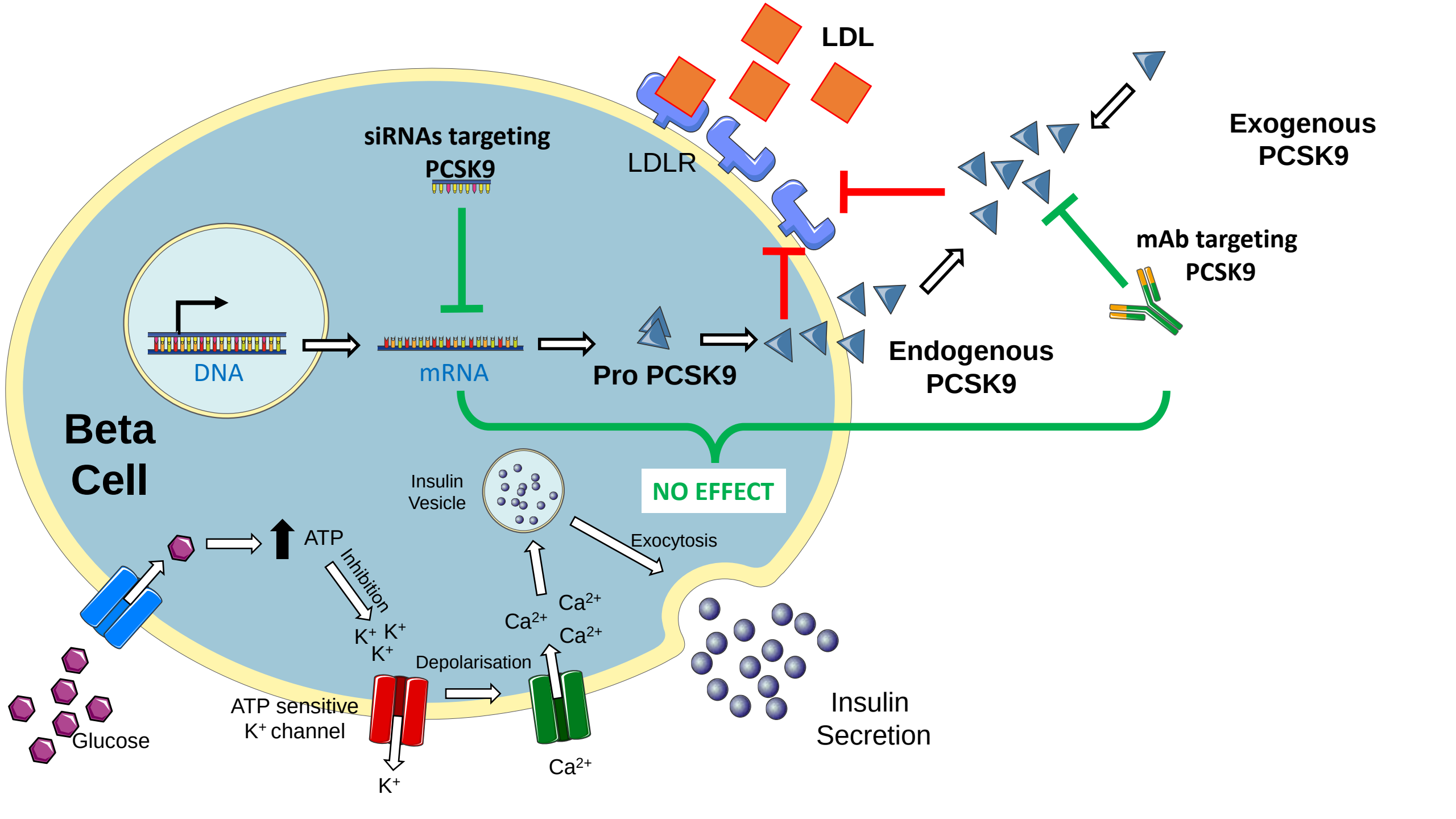
Merge
10 μm


A
PCSK9 secretion (ng/10^6 seeded cells)
25
20
15
10
5
0
**
No statin
Mevastatin
30
20
10
0
**
*
No statin
Simvastatin
Pravastatin
B
PCSK9 secretion (ng/200 IE)
0.5
0.4
0.3
0.2
0.1
0.0
**
*
No statin
Mevastatin
Donor 1
Donor 2


A
Cell surface LDLR expression (RMFI)
150
100
50
0
Mevastatin (10 μg/mL)
PCSK9 D374Y (600ng/mL)
Alirocumab (10 μg/mL)
Mevalonate (30 μg/mL)
B
Fluorescent LDL uptake (RMFI)
400
300
200
100
0
Mevastatin (10 μg/mL)
PCSK9 D374Y (600ng/mL)
C
a
b
c
d
5 μm
D
Fold change of PCSK9 secretion (relative to siRNA control)
1.5
1.0
0.5
0.0
siRNA control
siRNA PCSK9
E
Fold change of cell surface LDLR expression (relative to siRNA control)
1.5
1.0
0.5
0.0
siRNA control
siRNA PCSK9


A
GSIS
(fold induction)
8
6
4
2
0
Mevastatin
(10μg/mL)
PCSK9 D374Y
(600ng/mL)
- - + +
- + - +
B
Initial insulin content
(MicroU / well)
15000
10000
5000
0
Mevastatin
(10μg/mL)
PCSK9 D374Y
(600ng/mL)
- - + +
- + - +
C
Fold change of GSIS
(relative to control)
2.0
1.5
1.0
0.5
0.0
0 μg/mL mevastatin
10 μg/mL mevastatin
PCSK9 D374Y
(600ng/mL)
Alirocumab
(10μg/mL)
- - + +
- + - +
D
Fold change of GSIS
(relative to siRNA control)
2.0
1.5
1.0
0.5
0.0
p=0.45
siRNA Control
siRNA PCSK9
E
GSIS
(fold induction)
5
4
3
2
1
0
+ LDL (250g/dL)
*
*
##
*
Mevastatin
(10μg/mL)
PCSK9 D374Y
(600ng/mL)
- - - + +
- - + - +
F
Initial insulin content
(MicroU / well)
20000
15000
10000
5000
0
+ LDL (250g/dL)
Mevastatin
(10μg/mL)
PCSK9 D374Y
(600ng/mL)
- - - + +
- - + - +


LDL
LDLR
siRNAs targeting PCSK9
Exogenous PCSK9
mAb targeting PCSK9
DNA
mRNA
Pro PCSK9
Endogenous PCSK9
Beta Cell
NO EFFECT
Insulin Vesicle
Exocytosis
ATP
Inhibition
K+ K+ K+
Ca2+ Ca2+ Ca2+
Depolarisation
ATP sensitive K+ channel
Glucose
K+
Ca2+
Insulin Secretion