Elsevier Editorial System(tm) for Molecular and Cellular Endocrinology

Manuscript Draft

Manuscript Number:

Title: Secreted PDZD2, like GLP-1, exerts an insulinotropic effect on INS-1E cells by a PKA-dependent mechanism

Article Type: Research Paper

Keywords: PDZD2, secreted PDZD2, INS-1E, insulin, PKA, PDX1

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Manuscript Region of Origin: HONG KONG

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Research Data Related to this Submission
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There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

# Secreted PDZD2, like GLP-1, exerts an insulinotropic effect on INS-1E cells by a PKA-dependent mechanism

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**Keywords**
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**Abstract**

Secreted PDZD2 (sPDZD2) is a signaling molecule generated upon proteolytic processing of the multi-PDZ-containing protein PDZD2. Previous analysis of gene-trap mice deficient in the synthesis of full-length PDZD2, but not the secreted form, revealed a role of PDZD2 in the regulation of glucose-stimulated insulin secretion. Here, using the pancreatic INS-1E β cells as *in vitro* model, we showed that depletion of PDZD2/sPDZD2 by RNA interference suppressed the expression of β-cell genes *Ins1*, *Glut2* and *MafA* whereas treatment with recombinant sPDZD2 rescued the suppressive effect. Similar to GLP-1, sPDZD2 stimulated intracellular cAMP levels and activated β-cell gene expression in a PKA-dependent manner. Depletion of PDX1 inhibited the sPDZD2 insulinotropic effect, which could also be demonstrated in mouse islets. Interestingly, *Pdzd2* expression in INS-1E cells and pancreatic islets was modulated by changes in glucose levels. In summary, our findings are consistent with sPDZD2 serving a signaling function in regulating β-cell gene expression.

## 1. Introduction

Glucose is the major physiological stimulus in triggering insulin secretion as well as insulin synthesis. The homeostasis of glucose is principally regulated by the actions of α and β cells from the pancreatic endocrine islets. Dysfunction of β cells, in which insulin is synthesized, leads to diabetes resulting from insufficient or defective secretion of insulin (Muoio and Newgard, 2008; Rachdaoui, 2020). Understanding of the regulation of pancreatic β-cell functions is crucial to the treatment of diabetes (Vetere et al., 2014; Komatsu et al., 2013)

sPDZD2 (secreted PDZ-domain containing 2), a 37-kDa protein possessing two PDZ domains, is generated from caspase-dependent proteolytic cleavage at the carboxy terminus of PDZD2 (Yeung et al. 2003). This secreted protein is found to be highly conserved among several species, including human, rat and mouse. Interestingly, the carboxy terminal region of sPDZD2 shows extremely high homology to interleukin-16 (IL-16). Indeed, IL-16 is the only secreted protein found in the PDZ protein family in addition to sPDZD2, and it is produced, in a similar way to sPDZD2, by caspase-3-dependent cleavage of its precursor protein, pro-IL-16 (Zhang et al., 1998). Several studies reported that IL-16 acts as a signaling molecule in regulating proliferation and activation of lymphocytes and the release of pro-inflammatory cytokines (Cruikshank and Little, 2008; Richmond et al., 2014). It is plausible that sPDZD2 may be another secreted PDZ protein functioning as extracellular signaling molecule to carry out regulatory actions, specifically in the endocrine β cells in which it is expressed at a high level (Ma et al., 2006).

Previous studies demonstrated that recombinant sPDZD2 protein stimulates proliferation in pancreatic β-cell derived INS-1E cells under low-serum conditions (Ma et al., 2006) as well as induces mitogenic effects in pancreatic progenitor cells (PPCs; Suen et al., 2008). At higher concentration around $10^{-9}$ M, sPDZD2 promoted PPC differentiation, as evidenced by the upregulation of many pancreatic endocrine marker genes including *PDX1* and *INS* (Leung et al., 2009). Depletion of endogenous sPDZD2 levels could suppress this upregulation. However, analysis of two mouse gene-trap lines revealed increased glucose-stimulated insulin secretion but insulin production was maintained (Tsang et al., 2010). Further expression analysis indicated that one precursor form of PDZD2 (Thomas et al., 2009), but not the secreted form, was depleted in mutant mouse pancreases (Tsang, 2008). Thus, the gene-trap mouse model is not sPDZD2-null and the functional relationship between sPDZD2 and regulation of insulin expression remains unclear.

In this study, we suppressed *Pdzd2* expression using siRNAs in INS-1E cells. Depletion

of PDZD2/sPDZD2 led to downregulation of β−cell gene expression at both RNA and protein levels and decreased cellular insulin content. Importantly, the suppressive effect could be rescued by the addition of recombinant sPDZD2 to the culture medium. sPDZD2 displayed concentration-dependent effect on β−cell gene expression and the maximal effect was observed at $10^{-10}$ M. RNA synthesis inhibitor, but not protein synthesis inhibitor, could counteract the stimulatory effect, suggesting that regulation is at the transcriptional level. Knockdown of PDX1 expression was shown to abrogate the sPDZD2-stimulatory effect. GLP-1 is a well-known insulinotropic peptide hormone widely recognized for its biological actions on enhancing insulin gene transcription, stimulating proinsulin biosynthesis, expanding islet-cell mass, as well as protecting β cells from apoptosis (Lee and Jun, 2014; Andersen et al., 2018). Here, we demonstrated that sPDZD2 could enhance the insulinotropic effect of GLP-1 and that treatment with PKA inhibitor could suppress the insulinotropic effects of both factors. Interestingly, *Pdzd2* transcription was found to be regulated by glucose levels in both INS-1E and pancreatic islet cells, hinting a physiological role played by PDZD2/sPDZD2 in the regulation of glucose homeostasis.

## 2. Materials and methods

### *2.1. Cell culture and RNA interference*

INS-1E cells (passages 52-80) were cultured at 37°C under a humidified condition of 95% air and 5% $CO_2$ in RPMI-1640 medium containing 11 mM glucose and supplemented with 5% heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), 1 mM sodium pyruvate, 10 mM HEPES and 50 μM β-mercaptoethanol. For RNAi, the non-silencing duplex 1 (5'- UUCUCCGAACGUGUCACGUdTdT- 3' from Qiagen) was used as a negative control. Duplex 2 (5'- GGCAAGGGCCUUGGCUUUAdTdT- 3' from Qiagen) and duplex 3 (5'-GCUGCAGGAGUACAUCCAATT- 3' from Health & Co.) were designed for targeting the third and first PDZ domain, respectively, of *Pdzd2* transcripts. The *Pdx1* siRNA duplex was purchased from Qiagen. The siRNA duplexes were transfected into INS-1E cells using HiPerfect transfection reagent (Qiagen). Samples were harvested 48 h after transfection.

### *2.2 Mouse pancreatic islet isolation*

The isolation procedure was adapted from Li et al. (2009). Mouse was sacrificed by cervical dislocation and an incision was made at the abdomen to expose the liver and intestines. The ampulla of the duodenum was clamped with haemostatic forceps. Perfusion was performed by injecting 2ml ice-cold collagenase solution [1.5 mg/ml of collagenase XI (Sigma) in 1X Hank's balanced salt solution (HBSS)] slowly into the common bile duct using 30G x 1 inch needle inserted at the junction of the hepatic and cystic duct. The inflated, fluid-filled pancreas was excised out and further digested in collagenase solution for 12 min at 37 $^{o}$C with gentle shaking and then stopped with ice-cold stop solution (1 mM $CaCl_2$ in 1X HBSS). The digested pancreas was centrifuged at 200 x g for 2 min at 4 $^{o}$C. The pellet was resuspended in stop solution, centrifuged again before resuspension in stop solution and passed through a 70μm cell strainer to remove exocrine cells. The islets were rinsed with stop solution, followed by flushing out from the cell strainer and transferred to petri dish for tissue culture in RPMI-1640 medium.

### *2.3. Treatment with recombinant proteins and various drugs*

To explore the effects of sPDZD2, INS-1E cells ($4x10^4$) or mouse islets in groups of 15 were cultured in 24-well plates and incubated with or without sPDZD2 ($10^{-12}$ M - $10^{-8}$ M) in complete culture medium for 48 h. Recombinant sPDZD2 was prepared as previously reported (Ma et al., 2006). For studying the effect of GLP-1, cells were incubated with $10^{-8}$ M

GLP-1 (7-36) amide (Bachem) in complete culture medium for 48 h. To investigate the signaling pathway of sPDZD2, protein kinase A (PKA) inhibitor (KT7520 from Tocris) was used. INS-1E cells were incubated with 5 x $10^{-6}$ M of PKA in addition to $10^{-10}$ M sPDZD2 or $10^{-8}$ M GLP-1 for 48 h. Moreover, cells were incubated with $10^{-10}$ M sPDZD2 together with either actinomycin D ($10^{-9}$ M) (Calbiochem) or cycloheximide ($10^{-8}$ M) (Sigma) for 48 h to distinguish whether the sPDZD2-stimulated gene expression was regulated transcriptionally or translationally. In another set of experiments, $10^{-12}$ M sPDZD2 and $10^{-9}$ M GLP-1 were added simultaneously to the cells for 48 h to study their additive effects.

*2.4 RNA extraction and quantitative PCR analysis*

INS-1E cells or mouse islets were lysed using TRIzol reagent (Invitrogen) according to the manufacturer's instruction for RNA extraction. Total RNAs were reverse transcribed using random hexamers and SuperScript III Reverse Transcriptase (Invitrogen). For analysis of *Pdzd2, Ins2* and *Glut2* expression in INS-1E and mouse islets, cDNAs were mixed with gene specific primers (Supplementary Table 1) and SYBR® Select Master Mix (Thermo Fisher Scientific) and subjected to the thermal cycle program of the StepOne™ Real-Time PCR instrument (Thermo Fisher Scientific) as follows: 2 min at 50°C followed by 2 min at 95 °C and then 40 cycles of 15 s at 95°C and 1 min at 60°C. TaqMan-based qPCR reactions were performed to determine the gene expression level for rat *Ins1, Glut2, MafA, Pdx1* and *Gapdh* in INS-1E cells. cDNAs were mixed with TaqMan® Assay probe (ABI) (Supplementary Table 2) and 2X TaqMan® FAST Universal Master Mix (ABI) and subjected to the ABI 7500 Real-time PCR System for analysis as follows: 5 min at 95 °C followed by 40 cycles of 15 s at 95°C and 35 s at 60°C. Results were analysed by StepOne™ Software (Thermo Fisher Scientific) using relative quantification ($\Delta\Delta C_T$) method.

*2.5. Measurement of cellular insulin content and cAMP levels*

At the time of harvest, INS-1E cells in each well were washed three times with PBS, and extracted with 500 μL of acid ethanol (15 mM/L HCl in 75% ethanol) for 18 h at 4°C. After centrifugation at 13,000 rpm for 5 min at 4°C, the supernatant was collected for the measurement of cellular insulin content utilizing the insulin ELISA kit (LINCO Research). To measure intracellular cAMP levels, the cAMP direct immunoassay kit (Abcam) was used. Samples and standards were prepared according to manufacturer's instructions. cAMP levels in samples were corrected against the background measurement of assay buffer and calculated according to the calibration curve generated using cAMP standards.

*2.6. Total protein extraction, sub-cellular fractionation and immunoblot analysis*

Protein samples from INS-1E cells were either extracted using RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM EDTA, 50 mM Tris-Cl (pH 7.4), proteinase inhibitor cocktail (Roche Applied Science)] or the ProteoExtract Subcellular Proteome Extraction Kit (EMD Biosciences, Inc.) according to the manufacturer's instruction for different subcellular fractions. For western blot analysis, protein extracts were solubilized in 5X SDS sample buffer [62.5 mM Tris-Cl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 0.025% bromophenol blue, 20% glycerol] and boiled for 10 min. Equivalent amounts of protein (20 μg) were resolved on SDS polyacrylamide gels (7.5-15%), subjected to electrophoresis and transferred to Hybond-C membranes (Amersham). Bound proteins were probed with specific primary antibodies against PDZD2 (1:1000, USB), GLUT2 (1:500, Santa Cruz), PDX1 (1:500, Santa Cruz), MAFA (1:4000, Bethyl Lab.), LAMIN A (1:1000, Santa Cruz) and α-TUBULIN (1:1000, NeoMarkers), and subsequently with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Pierce).

*2.7. Immunofluorescence*

INS-1E cells were grown on poly-L-lysine-treated glass coverslips in complete medium with the addition of sPDZD2 protein for 48 h. At time of harvest, cells were fixed in ice-cold acetone/methanol (1:1 v/v) for 15 min, blocked with 3% BSA/PBS for 1 h, incubated with anti-PDX1 antibody for 1 h and then stained with FITC-conjugated anti-goat antibody for 30 min in the dark. All procedures were done at room temperature. The fluorescent-stained preparations were mounted onto slides with DAPI mounting fluid. Images were acquired using the confocal microscope LSM 510meta (Carl Zeiss).

*2.8. Statistical analysis*

Data were collected from at least 3 individual experiments and were presented as means ±S.E.M. Significance was evaluated by unpaired Student's t-test or one-way analysis of variance (ANOVA) with appropriate post-hoc tests. Statistical significance by either unpaired Student's t-test or ANOVA was explicitly identified by text or in the figure legends.

## 3. Results

### *3.1. Pdzd2-silencing in INS-1E cells suppresses Ins1 and Glut2 expression*

We have previously demonstrated using two gene-trap mouse lines that suppression of *Pdzd2* expression led to defects in insulin secretion (Tsang et al., 2010). However, we did not detect any appreciable difference in β−cell gene expressions in the *Pdzd2*-deficient mice (Tsang et al., 2010). Subsequent expression analysis indicated that viral insertion in the

gene-trap mutants did not interfere with sPDZD2 synthesis although a full-length precursor form of PDZD2 was abolished (Tsang, 2008). qPCR analysis indicated that transcription from an alternative promoter led to synthesis of another precursor form shPDZD2 (Thomas et al., 2009), which could still be processed to generate sPDZD2. The role of sPDZD2 in the regulation of pancreatic β–cell function remains unclear. To address this issue in the present study, we suppressed *Pdzd2* expression in INS-1E cells using two *Pdzd2*-targeting siRNA duplexes (duplex 2 and 3), with another non-targeting duplex (duplex 1) as control. As shown in Fig. 1A, depletion of PDZD2/sPDZD2 expression revealed significant decreases in the mRNA levels of *Ins1, Glut2, MafA* but not *Pdx1* using duplex 2, with duplex 3 showing a similar but weaker suppressive effect. Immunoblot analysis also indicated suppression at the protein level (Fig. 1B) and ELISA analysis of cellular insulin content reflected significant downregulation by ~25% (Fig. 1C).

*3.2. sPDZD2 stimulates β–cell gene expression in INS-1E cells*

Since sPDZD2 was previously known to promote PPC differentiation with activation of pancreatic endocrine marker gene expression (Leung et al., 2009), we hypothesized that sPDZD2 is the functional form that induces β–cell gene expression. To test this hypothesis, we depleted expression of both the precursor and secreted forms of PDZD2 by RNA interference and tested whether addition of recombinant sPDZD2 alone could rescue β–cell gene expression. As shown in Fig. 2, sPDZD2 at $10^{-10}$ M when added exogenously significantly increased mRNA and protein levels of various β–cell markers even when *Pdzd2* expression was depleted by duplex 2 (the stronger duplex). As negative control, heat-denatured sPDZD2 was also tested but there was no rescue of gene expression. Signaling proteins including sPDZD2 are known to show concentration-dependent effects (Ma et al., 2006). To determine the dose-dependent effect of sPDZD2 on β-cell gene expression, we treated INS-1E cells with sPDZD2 ranging from $10^{-12}$ to $10^{-8}$ M. sPDZD2 was found to be most effective at $10^{-9}$ to $10^{-10}$ M on stimulating *Ins1, Glut2 and MafA* mRNA levels whereas *Pdx1* levels remained unchanged (Fig. 3A). Consistent with sPDZD2 exerting its effect at the transcription level, co-treatment with RNA synthesis inhibitor (actinomycin D) but not protein synthesis inhibitor (cycloheximide) strongly suppressed the sPDZD2-mediated effect on β–cell gene expression (Fig. 3B). To address the physiological relevance of the sPDZD2 stimulatory effect, we treated mouse islets with sPDZD2 at $10^{-9}$ M for 2 days. qPCR analysis revealed upregulation of both *Ins2* and *Glut2* after sPDZD2 treatment (Supplementary Fig. 1).

PDX1 is a homeodomain-containing transcription factor well known to regulate the expression of both *Ins1, Glut2* and *MafA* (Gao et al., 2014; Zhu et al., 2017). To test the notion that sPDZD2 might exert its activating effect via PDX1, mRNA level of which was

unchanged by sPDZD2 treatment, we tested whether the sPDZD2-mediated effects on β−cell gene expression was abolished when *Pdx1* expression was suppressed using a commercially available *Pdx1* targeting duplex. As expected, downregulation of *Pdx1* expression at both mRNA and protein levels led to suppression of sPDZD2-mediated activation of *Ins1, Glut2* and *MafA* (Fig. 4).

*3.3. sPDZD2 acts synergistically with GLP-1 to activate β-cell gene expression*

The stimulatory effect of sPDZD2 on β-cell gene expression is reminiscent of GLP-1, which is another well-known insulinotropic factor (Holst, 2019). Since GLP-1 is known to act via G protein-coupled receptor leading to activation of adenylyl cyclase, increases in cAMP levels and stimulation of protein kinase A (PKA) (Graaf et al., 2016), we investigated whether the action of sPDZD2 is sensitive to the PKA inhibitor KT5720. KT5720 is recognized to bind the catalytic subunit of PKA in a competitive fashion against ATP and inhibits the kinase activity in a cAMP-independent manner (Davies et al., 2000). Importantly, treatment with KT5720 ($5 \times 10^{-6}$ M) significantly inhibited the insulinotropic effect of both sPDZD2 and GLP-1 (Fig. 5A), suggesting that sPDZD2, like GLP-1, acted via PKA. Indeed, treatment with sPDZD2 at various concentrations was found to induce cAMP levels intracellularly with forskolin used as positive control (Supplementary Fig. 2). As shown in Fig. 5B, GLP-1 treatment of INS-1E also exerted concentration-dependent effect and GLP-1 at $10^{-9}$ M was shown to activate *Ins1* sub-optimally and so was sPDZD2 at $10^{-12}$ M (see Fig. 3A). Interestingly, treatment with both factors at sub-optimal concentrations displayed an additive effect on the upregulation of both *Ins1*, *Glut2* and *MafA* (Fig. 5C), consistent with these signaling factors acting via a common PKA-dependent mechanism.

PDX1 was previously shown to be regulated by increased protein stability and nuclear translocation (Claiborn et al., 2010; Semache et al., 2013 and 2014). We argued that sPDZD2 treatment might similarly modify PDX1 post-translationally (without transcriptional activation as shown in this study). To test this hypothesis, we treated INS-1E cells with various concentrations of sPDZD2. Subcellular fractionation followed by immunoblot analysis revealed that sPDZD2 at around $10^{-9}$ M induced higher levels of PDX1 expression in the LAMIN A-marked nuclear fractions but not the TUBULIN-marked cytoplasmic fractions, suggesting stimulated nuclear translocation of PDX1 upon sPDZD2 treatment (Supplementary Fig. 3A). Further immunofluorescent analysis confirmed this increased nuclear import of PDX1 upon treatment of INS-1E cells with $10^{-9}$ M of sPDZD2 (Supplementary Fig. 3B).

*3.4. Pdzd2 expression in INS-1E cells and mouse islets is modulated by glucose levels*

Our findings indicated that sPDZD2 is a signaling molecule that regulates the expression of β–cell genes. If this effect is of physiological relevance, we suspect that endogenous *Pdzd2* expression might also be sensitive to changes in ambient glucose concentrations. To test this notion, INS-1E cells cultured in standard RPMI medium (with glucose at ~11mM) were exposed to RPMI medium containing low (2.8 mM) or high (13.8 mM) D-glucose for 2, 8, 16 or 24 h. As shown in Fig. 6A, qPCR analysis of *Pdzd2* expression showed a decreasing trend for high glucose treatment but the reverse for low glucose treatment. After high-glucose treatment, *Pdzd2* mRNA level decreased by 20% at both 16-h and 24-h as compared to 0-h control. For low-glucose treatment, *Pdzd2* expression increased by 40% and 60% at 16-h and 24-h, respectively, as compared to 0-h control. The response was due to the metabolic effects of glucose but not an osmolarity difference because adding L-glucose (2.8 mM D-glucose plus 11mM L-glucose) to make up the high glucose level did not lead to a suppression in *Pdzd2* expression (Fig. 6B). *Pdzd2* mRNA level stayed high similar to cells subjected to low-glucose treatment. Importantly, mouse islets incubated in low- or high-glucose medium for 24 h also displayed 30% difference in *Pdzd2* expression (Fig. 6C), suggesting that the modulatory effects of glucose on *Pdzd2* expression is of physiological relevance.

## 4. **Discussion**

Our previous study of PDZD2/sPDZD2 function using gene-trap mice revealed a role of PDZD2 in the regulation of glucose-stimulated insulin secretion (Tsang et al., 2010). However, further expression analysis confirmed defective expression of a precursor form of PDZD2 but not the processed secreted form of PDZD2 in these mice. PDZD2-immunoreactivity was first found to be localized specifically to the β cells of pancreatic islets, rather than the peripheral α cells or the abundant exocrine acinar cells (Ma

et al. 2006). Consistent with the primarily ER localization of PDZD2-immunoreactivity indicative of proteolytic processing to generate the secreted form, sPDZD2 was found to be expressed and secreted into the conditioned medium by INS-1E cells. sPDZD2 at concentration from $10^{-12}$ M to $10^{-10}$ M was found to have a mitogenic effect on INS-1E cells grown under low-serum conditions (0.5 % and 2% FBS). The mitogenic effect was lost when cells were grown under normal serum-containing medium (with 10% FBS). In contrast, we showed in this study that sPDZD2 at $10^{-9}$ M to $10^{-11}$ M stimulated the expression of *Ins1*, *Glut2* and *MafA*, which are critical genes required for maintaining β–cell identity under the control of PDX1 (Gao et al., 2014). The sPDZD2-stimulatory effect was exercised at the transcriptional level as treatment with RNA synthesis inhibitor could inhibit the effect and sPDZD2 was found to stimulate *Ins1* promoter activity in transient reporter assay (Tsang, 2008). Interestingly, *Pdx1* mRNA level was unaffected by sPDZD2 treatment but knockdown of *Pdx1* expression using siRNA abolished the sPDZD2 stimulatory effect, suggesting that sPDZD2 exerts its effect via modulating PDX1 function post-transcriptionally.

It is important to note that depletion of PDZD2/sPDZD2 expression in INS-1E cells led to suppression of *Ins1*, *Glut2* and *MafA*, but not *Pdx1* expression, at both RNA and protein levels. PDZD2/sPDZD2 depletion also lowered cellular insulin content. When sPDZD2 was supplemented as recombinant protein to the siRNA-treated cells, expression of the β genes was restored, supporting that the insulinotropic effect was mediated via the secreted form of PDZD2. Deficiency of expression of the precursor but not the secreted form of PDZD2 in the gene-trap mice reported earlier could explain why there was lack of defective islet structure/function in these hypomorphic mice (Tsang et al. 2010). Our recent attempt to establish *Pdzd2* null (ablating both the full-length and secreted forms) using the CRISPR/Cas9 strategy suggested that *Pdzd2* function is needed early in embryonic development (Tsui, 2013). No viable homozygous mice could be harvested as early as E9.5. Further inducible/tissue-specific knockout mice would need to be generated before the β cell-specific function of sPDZD2 could be confirmed *in vivo*.

We believe that the insulinotropic effect of sPDZD2 demonstrated in this study is of physiological relevance. In fact, sPDZD2 was found to promote PPC differentiation by stimulating *PDX1* and *INS* expression and depletion of endogenous sPDZD2 levels was found to suppress this effect (Leung et al., 2009). In the present study, we demonstrated that treatment of isolated mouse islets using recombinant sPDZD2 protein stimulated expression of both *Glut2* and *Ins2*. GLP-1 is another insulinotropic peptide hormone which is widely recognized for its biological actions on enhancing insulin gene transcription, stimulating proinsulin biosynthesis, expanding islet-cell mass, as well as protecting β cells from apoptosis (Lee and Jun, 2014; Andersen et al., 2018). Therefore, the GLP-1 peptide and its

derivatives have been regarded as ideal candidates for the treatment of diabetes (Holst, 2019). Comparable to GLP-1, sPDZD2 was found to stimulate cAMP levels and require PKA to mediate its effect. Treatment with the PKC inhibitor GF109203X did not show appreciable effect (results not shown). Importantly, co-treatment of sPDZD2 and GLP-1 at sub-optimal concentrations stimulated *Ins1*, *Glut2* and *MafA* expression, suggestive of an additive effect mediated via cAMP/PKA-dependent mechanism. However, identity of the putative G protein-coupled receptor of sPDZD2 remains unclear and its future identification and characterization would facilitate screening of drugs for enhancing β–cell function.

Similar to GLP-1, sPDZD2 exerted its insulinotropic effects via increasing the levels of nuclear PDX1. GLP-1 modifies PDX1 in a post-translational manner and ultimately regulates the expression of insulin (Wang et al. 2001). In fact, PDX1 needs to be stabilized and translocated to the nucleus to initiate its transcriptional regulatory capability through binding to promoters of β-cell genes, including *Insulin* and *Glut2* (Elrick and Docherty, 2001; Shao et al., 2009; Semache et al., 2013 and 2014). PDX1 nuclear translocation was suggested to be initiated by phosphorylation (Elrick and Docherty, 2001; An et al., 2010) and/or sumoylation (Kishi et al., 2003). Otherwise, non-stabilized PDX1 is degraded by proteasomes (Lebrun et al., 2005). Our study demonstrated that sPDZD2 enhances the nuclear localization of PDX1; however, its participation in the phosphorylation or sumoylation of PDX1 remains to be explored. Notwithstanding, further study of sPDZD2 as enhancer of GLP-1 function has definite implications for the treatment of diabetes.

sPDZD2 as physiological regulator of glucose homeostasis is also hinted by the modulation of endogenous *Pdzd2* expression by exogenous glucose levels. Low glucose levels were found to increase *Pdzd2* mRNA level in INS-1E cells and high-glucose treatment in mouse islets suppressed *Pdzd2* expression. The presence of serum in cultured cells and in blood hampers the monitoring of sPDZD2 levels by immunoblot analysis. Future design of sensitive ELISA for the detection of sPDZD2 *in vivo* is urgently needed to further elucidate the physiological role of sPDZD2. It is interesting to note that *Pdzd2* transcript level was found to be downregulated in five adipocyte-based models of insulin resistance (Lo et al., 2013). It remains to be determined whether sPDZD2 is up- or down-regulated in the conditioned medium of these models. Recent bioinformatics analysis indicates that PDZD2/sPDZD2 is expressed in multiple tissues/cell types including adipocytes. It would not be surprising that sPDZD2 may serve an endocrine function involved in multiple aspects of regulation of glucose homeostasis/insulin sensitivity.

In summary, our findings provide the first evidence to show that sPDZD2, like GLP-1, exerts an insulinotropic effect on β-like INS-1E cells via a PKA-dependent mechanism to

modulate PDX1 function. Further study of the regulation and downstream signaling effects of sPDZD2 is required to reveal its possible role in the regulation and treatment of diabetes.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Acknowledgements

This work was supported by a donation from Chi Lin Kok Ng Buddhist Hall limited to K.-M. Yao to study the molecular basis of cancer and diabetes. M. G. T. is a postdoctoral fellow supported by the Health and Medical Research Fund (Project No.: 06172876) awarded to K.-M. Yao by the Hong Kong Food and Health Bureau.

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**Figure legends**

**Figure 1. PDZD2 knockdown using siRNAs led to suppression of β-cell gene expression.** (A) *Pdzd2* in INS-1E cells was knocked down using siRNA duplex 2 and 3, which targets PDZ3 and PDZ1 domain of PDZD2, respectively, for 48 h. Duplex 1 was used as

non-silencing control. Both duplex 2 and 3 led to suppression of the β-cell genes, *Ins1, Glut2* and *MafA*, but not *Pdx1* at mRNA level. Gene expression levels were normalized against *Gapdh* and expressed as fold differences when compared against the untreated control. (B) A representative blot from 4 individual experiments. Western blotting showed that PDZD2 was depleted more effectively by duplex 2 than duplex 3 at protein level, but not by duplex 1 control. Cells were harvested 72 h after transfection. (C) Suppression of *Pdzd2* expression by RNA interference led to reduced cellular insulin content in INS-1E measured by insulin ELISA assay after 48 h. Cellular insulin contents were expressed as fold changes when compared against the non-silencing duplex 1 control. Data are means ± S.E.M. of values from 6 (A) and 3 (C) individual experiments, *$p < 0.05$.

**Figure 2. Suppression of β-cell gene expression could be rescued by addition of recombinant sPDZD2.**

(A) qPCR analysis showed that the suppressive effect on β genes *Ins1, Glut2* and *MafA* by knockdown of *Pdzd2* using siRNA duplex 2 was significantly rescued by co-treatment with exogenous recombinant sPDZD2 ($10^{-10}$ M) for 48 h. Denatured sPDZD2 served as negative control and displayed no rescue effect. Gene expression levels were normalized against *Gapdh* and expressed as fold differences when compared against the untreated control. Data are means ± S.E.M. of values from 6 individual experiments, *$p < 0.05$. (B) A representative blot from 4 individual experiments. Immunoblot analysis showed that the suppressive effect on GLUT2 and MAFA expression was counteracted at protein level after sPDZD2 co-treatment for 72 h.

**Figure 3. Recombinant sPDZD2 stimulated β-cell gene expression at the transcriptional level.**

(A) qPCR analysis showed that addition of recombinant sPDZD2 stimulated the expression of *Ins1, Glut2* and *MafA*, but not *Pdx1* at mRNA level in a concentration-dependent manner in INS-1E after 48 h, with the highest stimulatory effect achieved at $10^{-10}$ M. (B) Co-incubation of $10^{-10}$ M sPDZD2 with actinomycin D ($10^{-9}$ M), but not cycloheximide ($10^{-8}$ M) for 48 h significantly abolished the stimulatory effects of sPDZD2 on the β-cell genes expression. Gene expression levels were normalized against *Gapdh* and expressed as fold differences when compared against the untreated control. Data are means ± S.E.M. of values from 3(A) and 6 (B) individual experiments, *$p < 0.05$, P*** $< 0.01$.

**Figure 4. PDX1 knockdown abrogated sPDZD2-stimulated β-cell gene expression.**

Depletion of PDX1 abrogated the stimulatory effect of exogenous sPDZD2 ($10^{-10}$ M) on β-cell gene expression at both mRNA (A) and protein (B) levels. (A) qPCR analysis showed that knockdown of *Pdx1* using a specific siRNA duplex, but not the control siRNA,

suppressed the expression of *Ins1, Glut2* and *MafA* mRNAs in INS-1E after 48 h. Gene expression levels were normalized against *Gapdh* and expressed as fold differences when compared against treatment with the control duplex. Data are means ± S.E.M. of values from 6 individual experiments, *$p < 0.05$. (B) Representative blot from 3 individual experiments.

**Figure 5. sPDZD2, like GLP-1, exerted PKA-dependent stimulatory effect on β-cell gene expression.**

(A) qPCR analysis showed that addition of exogenous GLP-1 ($10^{-8}$ M) could induce *Ins1*, *Glut2* and *MafA* mRNA expression, analogous to the stimulatory effect of sPDZD2 ($10^{-10}$ M) on INS-1E after 48 h. The stimulatory effect on β-cell genes was abolished for both sPDZD2 and GLP-1 when 5 x $10^{-6}$ M of PKA inhibitor (KT5720) was added. (B) Like sPDZD2, GLP-1 exerted a stimulatory effect on *Ins1* expression in a concentration-dependent manner, with $10^{-8}$ M showing the most significant effect. (C) Co-incubation of GLP-1 ($10^{-9}$ M) and sPDZD2 ($10^{-12}$ M) (both at sub-optimal dose) for 48 h in INS-1E showed additive stimulatory effects on the mRNA expression of *Ins1, Glut2* and *MafA*. Gene expression levels were normalized against *Gapdh* and expressed as fold differences when compared against the untreated control. Data are means ± S.E.M. of values from 6 individual experiments for both A, B and C, *$p < 0.05$.

**Figure 6. *Pdzd2* transcription was subjected to regulation by glucose levels.**

(A) *Pdzd2* mRNA expression levels were analyzed by qPCR analysis in INS-1E cells incubated with low (2.8mM) or high (13.8mM) glucose medium for 2, 8, 16 or 24 h. (B) INS-1E was incubated with low (2.8mM), high (13.8mM) glucose medium, or osmotic control (2.8mM D-glucose plus 11mM L-glucose) for 24 h. qPCR analysis showed that the changes in *Pdzd2* expression were not due to a difference in osmolarity. (C) Isolated mouse pancreatic islets were incubated with low (2.8mM) or high (13.8mM) glucose medium for 24 h before their harvest for analysis of *Pdzd2* mRNA level. *Pdzd2* mRNA levels were normalized against *Actb* and expressed as percentage change when compared against the 0-h control (A and B). For C, *Pdzd2* expression levels were normalized against 18S ribosomal RNA and compared against mouse islets incubated in high-glucose medium. Data are means ± S.E.M. of values from 3 individual experiments for A, B and C, *$p< 0.05$, **$p < 0.01$, ***$p< 0.001$, ns, not significant.

**Supplementary Figure 1. sPDZD2 stimulated *Ins2* and *Glut2* expression in mouse islets.**

Isolated mouse pancreatic islets were incubated in medium with or without $10^{-9}$ M sPDZD2 for 48 h. qPCR analysis showed that both *Ins2* and *Glut2* mRNA levels were induced by sPDZD2 treatment. Gene expression levels were normalized against 18S rRNA and expressed as percentage change when compared against the untreated control. Data are means ± S.E.M.

of values from 3 individual experiments, *p< 0.05.

**Supplementary Figure 2. sPDZD2 elevated intracellular cAMP levels.**
Total intracellular cAMP levels were measured in INS-1E cells upon sPDZD2 ($10^{-10}$ – $10^{-7}$ M) or forskolin ($10^{-10}$ – $10^{-7}$ M) treatment at different concentrations for 30 min, in the presence of 0.5mM IBMX in serum-free RPMI medium. cAMP concentrations were compared to the untreated control. Data are means ± S.E.M. of values from 3 individual experiments, *p< 0.05, **p<0.01, ***p< 0.001.

**Supplementary Figure 3. Increased nuclear localization of PDX1 upon sPDZD2 stimulation.**
(A) Whole cell lysates of INS-1E cells treated with different concentrations of sPDZD2 ($10^{-11}$, $10^{-9}$ and $10^{-7}$ M) for 48 h were separated into nuclear (indicated by LAMIN A expression) and cytoplasmic (indicated by TUBULIN expression) fractions. Western Blotting showed increased nuclear localization of PDX1 in the presence of sPDZD2. (B) Immunostaining of INS-1E using anti-PDX1 antibody with DNA labelled with DAPI (blue) showed consistent nuclear localization of PDX1 protein (green) after treatment with $10^{-9}$ M of sPDZD2 for 48 h.

**Figure 1**

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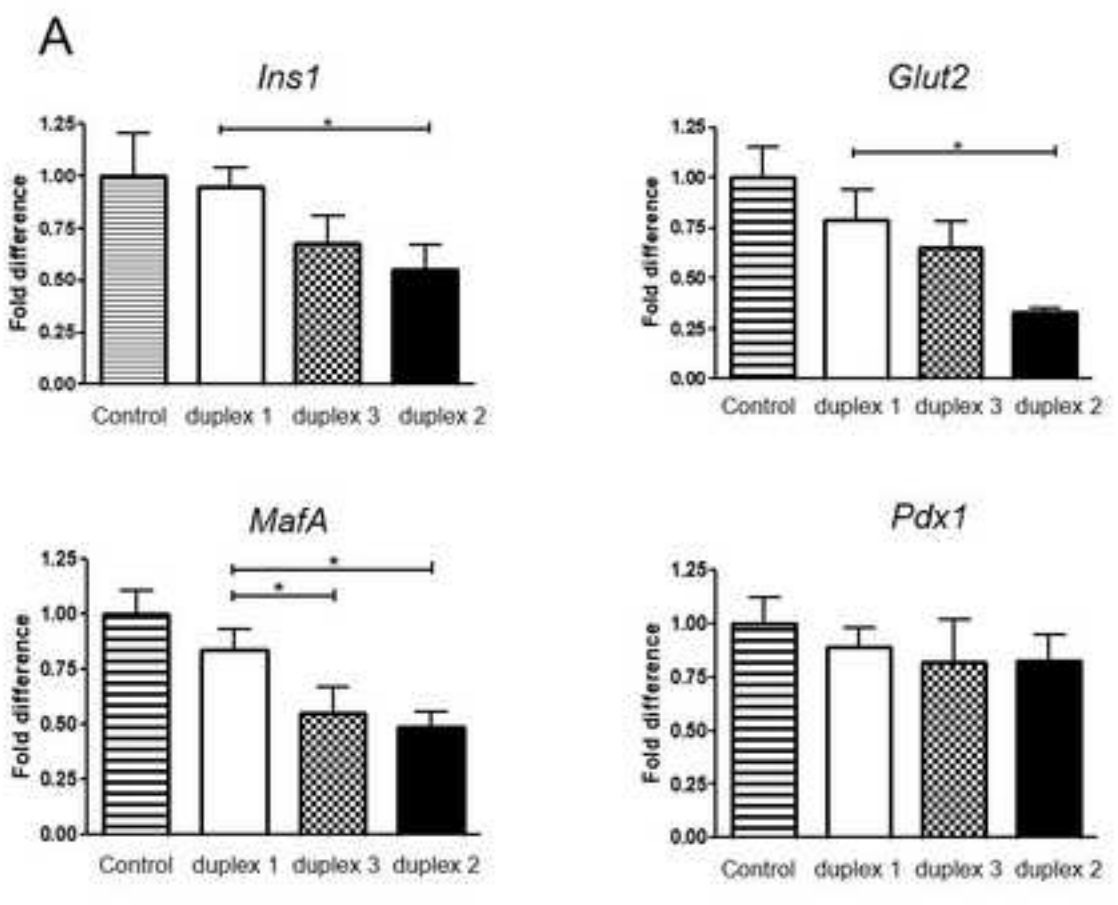


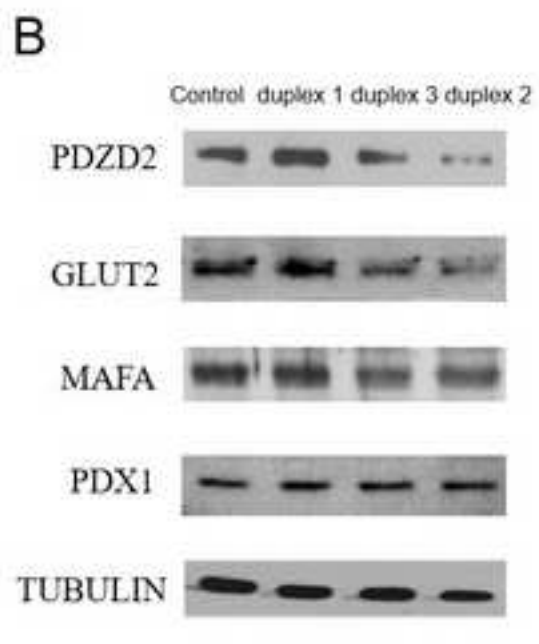


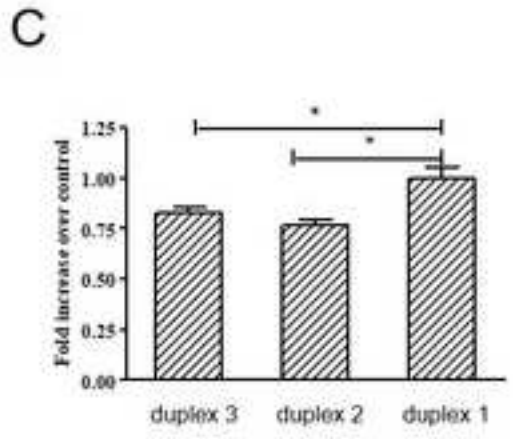

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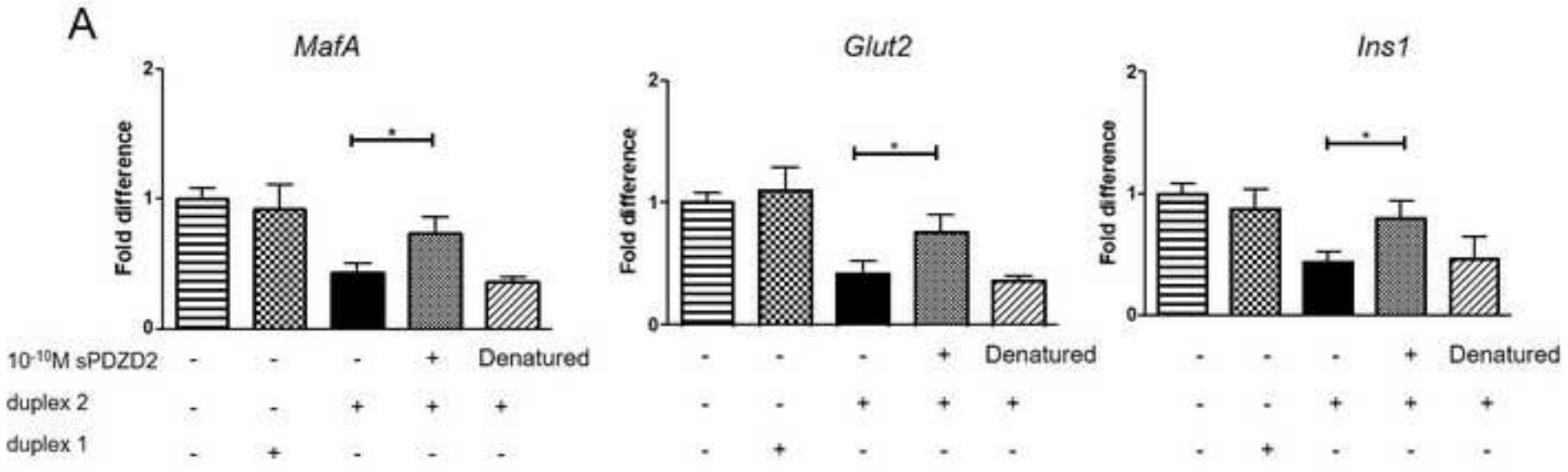


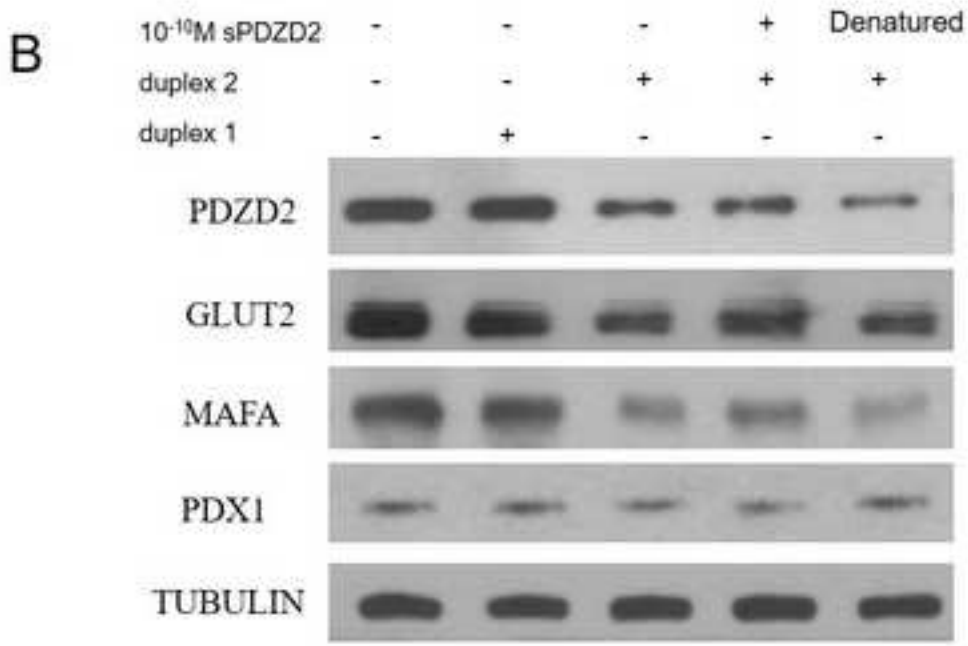

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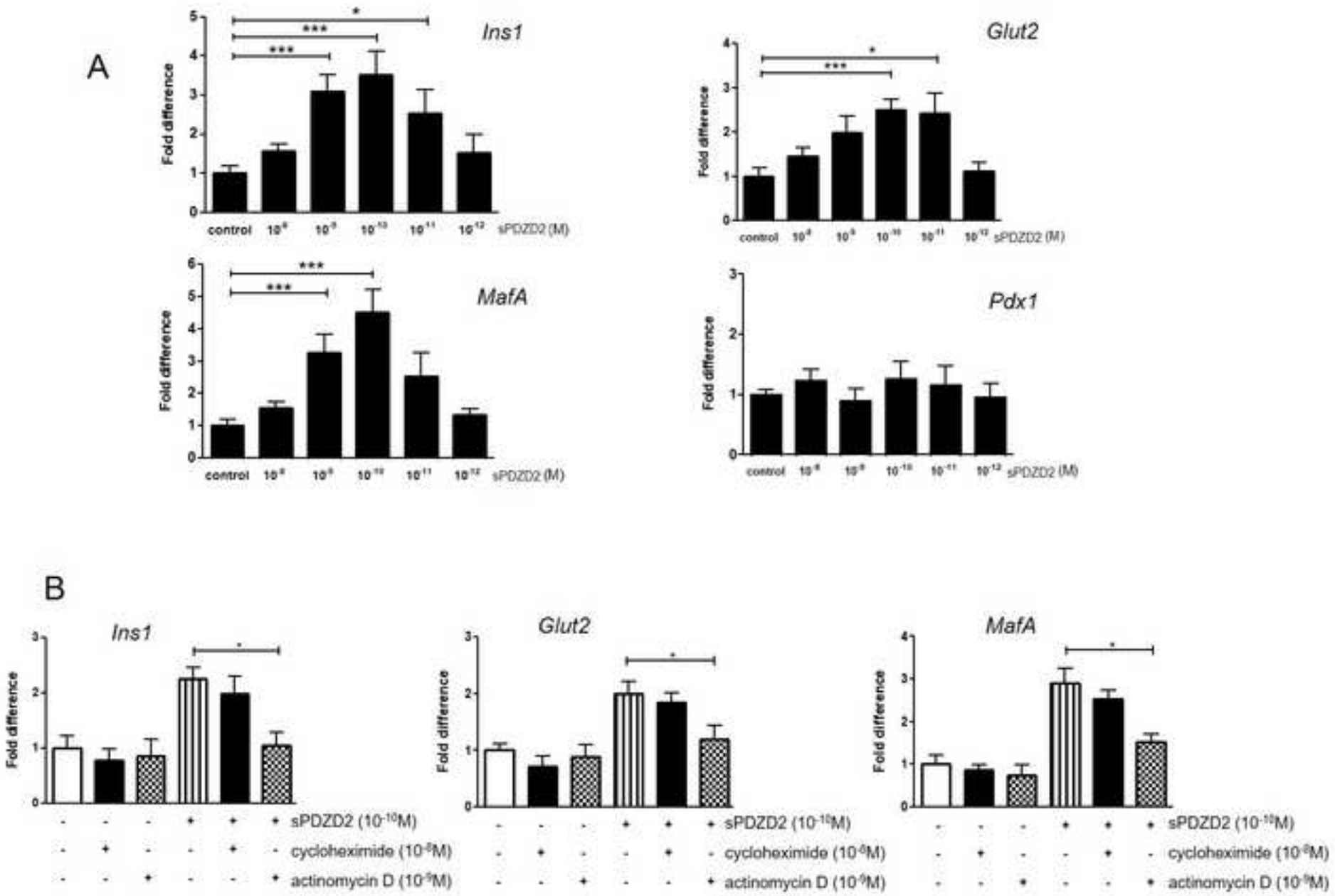

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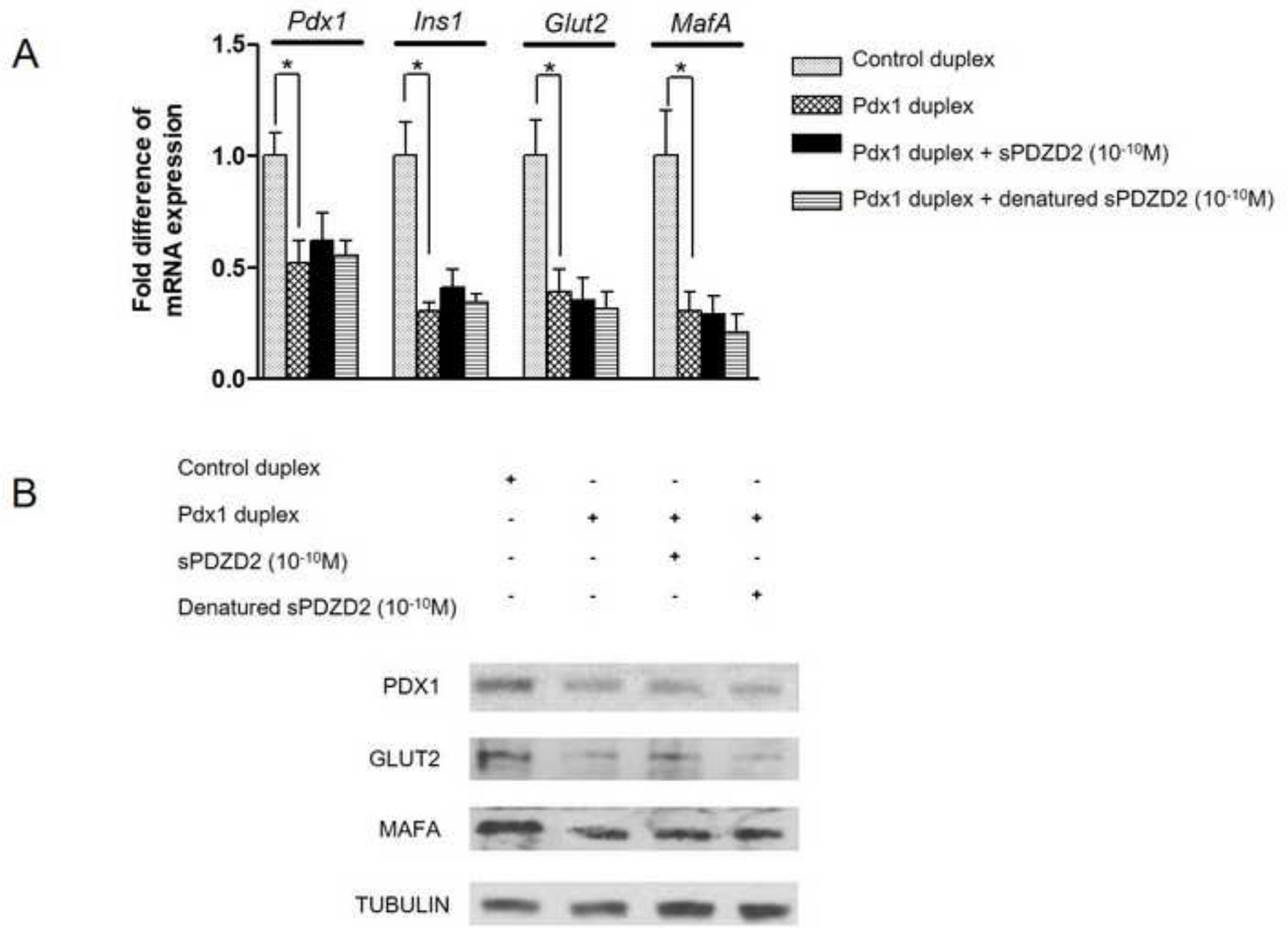

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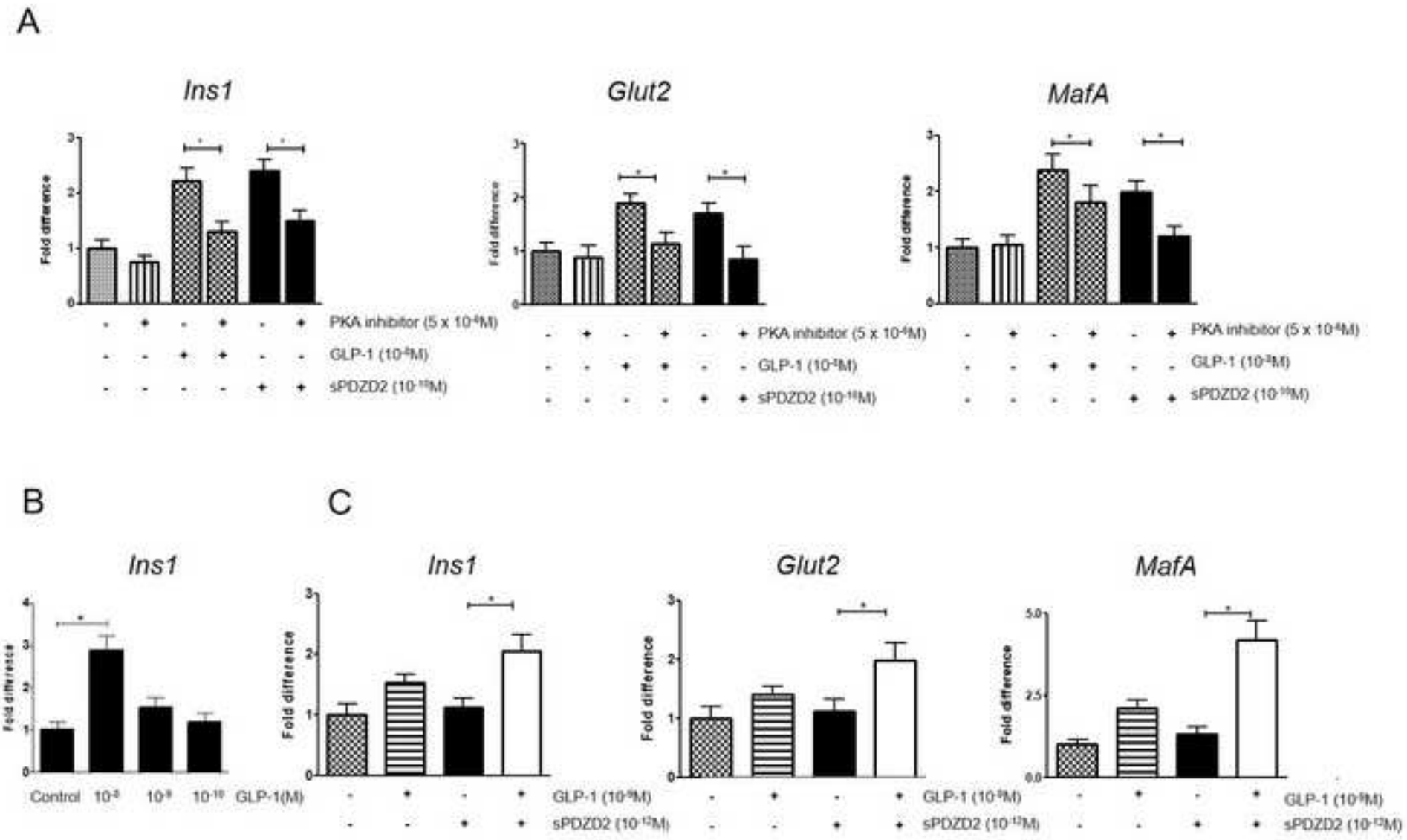

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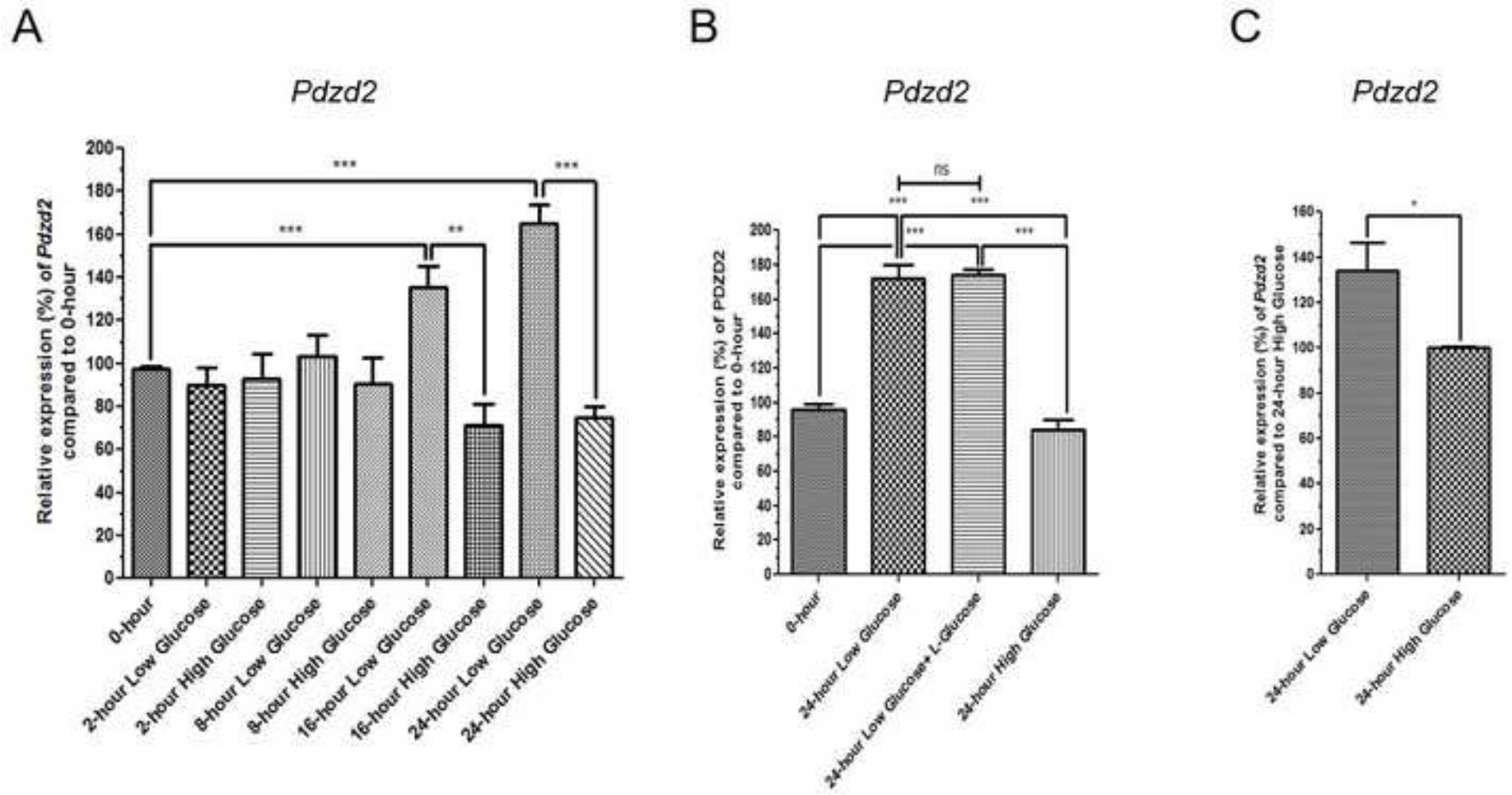

**Supplementary Figure 1**

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**Supplementary Table 2**

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Credit Author Statement

K-MY conceptualized and supervised this research work. He acquired funds for supporting this study. DHFS, JCYC and PSWT conducted the research work and generated the data. MGT curated the data and prepared the Figures and Tables. K-MY wrote this paper with the assistance of PSWT, MGT and DHFS.