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Morphological and functional adaptations of pancreatic alpha-cells during late pregnancy in the mouse.

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Abstract

Background: Pregnancy represents a major metabolic challenge for the mother, and involves a compensatory response of the pancreatic beta-cell to maintain normoglycaemia. However, although pancreatic alpha-cells play a key role in glucose homeostasis and seem to be involved in gestational diabetes, there is no information about their potential adaptations or changes during pregnancy.

Material and methods: Non-pregnant (controls) and pregnant C57BL/6 mice at gestational day 18.5 (G18.5) and their isolated pancreatic islets were used for *in vivo* and *ex vivo* studies, respectively. The effect of pregnancy hormones was tested in glucagon-secreting α-TC1.9 cells. Immunohistochemical analysis was performed in pancreatic slices. Glucagon gene expression was monitored by RT-qPCR. Glucagon secretion and plasma hormones were measured by ELISA.

Results: Pregnant mice on G18.5 exhibited alpha-cell hypertrophy as well as augmented alpha-cell area and mass. This alpha-cell mass expansion was mainly due to increased proliferation. No changes in alpha-cell apoptosis, ductal neogenesis, or alpha-to-beta transdifferentiation were found compared with controls. Pregnant mice on G18.5 exhibited hypoglucagonaemia. Additionally, *in vitro* glucagon secretion at low glucose levels was decreased in isolated islets from pregnant animals. Glucagon content was also reduced. Experiments in α-TC1.9 cells indicated that, unlike estradiol and progesterone, placental lactogens and prolactin stimulated alpha-cell proliferation. Placental lactogens, prolactin and estradiol also inhibited glucagon release from α-TC1.9 cells at low glucose levels.

Conclusions: The pancreatic alpha-cell in mice undergoes several morphofunctional changes during late pregnancy, which may contribute to proper glucose homeostasis. Gestational hormones are likely involved in these processes.

Keywords: pregnancy, pancreatic alpha-cell, glucagon, pregnancy hormones.

Abbreviations: GDM, gestational diabetes mellitus; G, glucose; INS, insulin; PL, placental lactogen; PRL, prolactin; P, progesterone; E_2, estradiol.

1. Introduction.

To fulfill the energy requirements of the growing fetus, major physiological adaptations occur in the gravid mother. During late pregnancy, maternal insulin resistance in peripheral tissues allows for an adequate glucose gradient and supply to the fetus [1,2]. Among other factors, increased levels of gestational hormones are known to play a role in the decline of insulin sensitivity during late pregnancy [1]. Under this particular scenario, pancreatic islets undergo multiple adaptations driven predominantly by gestational hormones: placental lactogen (PL), prolactin (PRL), progesterone (P) and estradiol (E_2) [3]. These adaptations constitute a physiological response to insulin resistance in order to increase plasma insulin levels and maintain a normoglycemic state in the mother. Adaptive changes in pancreatic beta-cells involve hyperplasia, hypertrophy and increased secretory activity in both humans and rodents [1,2,4]. When these maternal beta-cell adaptations are not able to compensate for insulin resistance, hyperglycaemia and gestational diabetes mellitus (GDM) arises and leads to negative outcomes in the mother and offspring. GDM increases the maternal risk of developing Type 2 diabetes mellitus (T2DM) after delivery [1]. Moreover, GDM could also have adverse metabolic consequences for the offspring, such as impaired development of the endocrine pancreas, predisposition to obesity, glucose intolerance and T2DM later in life [5].

In addition to insulin, glucagon release from pancreatic alpha-cells also regulates glucose homeostasis [6]. Hypoglycemia stimulates secretion from these endocrine cells, leading to a rise in plasma glucagon levels and glucagon-induced hepatic glucose production to normalize glycaemia [6,7]. In recent years, accumulated evidence indicates that alterations in both glucagon secretion and pancreatic alpha-cell mass and function are involved in the development of hyperglycaemia and the etiopathogenesis of

diabetes [6,7]. Despite the importance of this islet cell population, the potential adaptations of the pancreatic alpha-cell during pregnancy are essentially unknown. Glucagon seems to play a significant role in the metabolism of placental glycogen cells [8,9] and the lack of glucagon signaling leads to fetoplacental defects and alterations in the maternal metabolic milieu in pregnant mice [10]. Like insulin, plasma glucagon levels exhibit dynamic changes throughout gestation in humans and mice [11,12]. It has been reported that the counter-regulatory glucagon response to hypoglycemia is diminished in pregnancy [13]. Remarkably, GDM is associated with higher plasma glucagon levels in late pregnancy [14] and with the lack of glucagon suppression in response to glucose [15], which can contribute to hyperglycemia. Despite these observations, virtually nothing is known about either the alterations or adaptations of the alpha-cell during gestation or the potential contribution of pregnancy hormones in pancreatic alpha-cell regulation. In the present study, we show that the pancreatic alpha-cell also undergoes several morphological and functional changes during pregnancy and that prolactin and placental lactogens may be involved in these alterations.

2. Materials and Methods.

2.1 Animals.

The procedures used in this work were previously evaluated and approved by the Animal Ethics Committee of the Miguel Hernandez University (UMH) in accordance with current national and European legislation. Animals were 8-10 weeks-old C57BL/6 female mice, which were supplied by the UMH Animal Experimentation Service. The animals were kept under controlled and standardized conditions with a light/dark cycle of 12 hours, 22ºC and *ad libitum* access to food and water. Non-pregnant female mice were established as controls, while the study group included pregnant mice on gestational days G12.5, G15.5 and G18.5. Mating was confirmed by the presence of a vaginal plug and this day was established as G0.5.

2.2 Cell culture.

For the *in vitro* experiments, we used the glucagon-releasing cell line α-TC1.9 (ATCC, Manassas, VA, USA). According to the supplier, these cells were derived from an adenoma, which was obtained in transgenic mice expressing the SV40 T antigen oncogene under the control of the rat preproglucagon promoter. Because of the similar secretory profile of α-TC1.9 cells to that of primary mouse alpha-cells, this cell line has been frequently used as a model to study calcium signaling and glucagon release from pancreatic alpha-cells [16]. α-TC1.9 cells were grown in DMEM (Invitrogen, Barcelona, Spain) without phenol red and supplemented with 2 mM l-glutamine, 1.5 g/l $NaHCO_3$, 10% inactivated FBS, 15 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mM non-essential amino acids, and a final glucose concentration of 3 g/l. Cells were treated for 8 days with the different pregnancy hormones: PL (500

ng/ml), PRL (500 ng/ml), P (100 ng/ml) and E_2 (100 pM). DMSO was used as vehicle. The media was refreshed every 48 hours.

2.3 Immunocytochemistry and immunohistochemistry.

For the immunohistochemistry, the pancreases were removed and fixed overnight in 4% paraformaldehyde. Subsequently, the tissue was embedded in paraffin and the sections were prepared for immunohistochemistry to identify glucagon-containing cells. After dehydration, the sections were heated to 100°C in the presence of citrate buffer (10mM, pH 6.0) for 20 min. Endogenous peroxidase was blocked by incubation with a solution of 3% hydrogen peroxidase in 50% methanol for 30 min. The sections were then incubated in 3% BSA in PBS for 1 h at room temperature to block nonspecific binding [17-19]. Two to three pancreas sections separated by 200 μm were measured per animal. The total pancreatic area, alpha-cell area and cell size were measured using Metamorph Analysis Software (Nashville, TN, USA). In these experiments, glucagon-containing cells were identified using a polyclonal anti-glucagon rabbit antibody (1:100; Monosan, Uden, The Netherlands) as previously described [17-19] with a hematoxylin counterstain to identify nuclei. Proliferation was analyzed using a monoclonal anti-Ki67 rabbit antibody (1:400; Cell Signaling Technology, Danvers, MS, USA) and a monoclonal anti-glucagon mouse antibody (1:1000; Sigma, Madrid, Spain) to identify the alpha-cells in hematoxylin-counterstained samples. A kit for a double immunostaining was used in this experiment (EnVision G|2 Doublestain System, Rabbit/Mouse; DAB+/Permanent Red) (Agilent DAKO, Santa Clara, CA, USA). In both experiments, images were acquired using a Nikon Eclipse TE200 microscope (20X objective). Apoptotic cell counting was analyzed in pancreas sections with the TUNEL technique [18,19]. Images were acquired using a Zeiss Axiovert 200 fluorescence

138 microscope (40X objective). Ductal neogenesis was analyzed using the monoclonal
139 anti-pan-Cytokeratin mouse antibody (1:300; Santa Cruz Biotechnology, Dallas, TX,
140 USA), as previously described [18]. In this case, images were acquired using a Zeiss
141 Axio Observer Z1 microscope with ApoTome (40X objective). To identify glucagon
142 and insulin double-positive cells, a mouse monoclonal anti-glucagon antibody (1:100;
143 Sigma, Madrid, Spain) and a rabbit anti-insulin antibody (1:100; Santa Cruz
144 Biotechnology, Dallas, TX, USA) were used. Double-positive cells were manually
145 counted using the LAS X software (Leica Microsystems Inc. Buffalo Grove, IL, USA).
146 Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG were
147 used as secondary antibodies in the apoptosis and neogenesis experiments, while Alexa
148 Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 goat anti-mouse IgG (1:500; Life
149 technologies, Carlsbad, CA, USA) were used in the immunostaining of glucagon-insulin
150 positive cells. Nuclei were stained with Hoechst 33342 (1:1000; Invitrogen, Barcelona,
151 Spain). Sections were mounted using ProLong Gold Antifade Reagent (Invitrogen,
152 Barcelona, Spain).

153 To study the proliferation rate in α-TC1.9 cells, the cells were seeded on
154 coverslips treated with poly-L-lysine (at least 100.000 cells/coverslip) and exposed for 8
155 days with the different hormones. The incubation medium was refreshed every 48
156 hours. On day 7, cells were incubated with 10 µM BrdU for 24 hours. Cells were then
157 fixed with 4% paraformaldehyde and immunofluorescence was performed. First, cells
158 were immersed in 70% ethanol at 4ºC for 30 min; then, cells were immersed in 2 N HCl
159 for 20 min, followed by incubation in a 0.1 M borax solution for 15 min at room
160 temperature and by a wash step after incubation with PBS. To prevent non-specific
161 binding, the cells were incubated for 1 h in 3% BSA in PBS at room temperature.
162 Staining was performed using monoclonal anti-BrdU mouse antibody (1:100; Agilent

Dako, Santa Clara, CA, USA) and propidium iodide to label the nuclei. The samples were mounted using ProLong Gold Antifade Reagent (Invitrogen, Barcelona, Spain). Images were acquired using a Zeiss LSM 510 confocal microscope (40X objective).

2.4 Plasma measurements.

Blood glucose was measured from the tail vein with an automatic glucometer (Accu-Chek Compact plus; Roche, Mannhein, Germany). Blood samples were collected from the saphenous vein using Microvette tubes (Sarstedt, Germany). For glucagon measurements, blood samples were supplemented with aprotinin (20 mg/l) to protect them from proteolysis. Plasma glucagon and insulin concentrations were determined by ELISA (Crystal Chem Inc., Elk Grove Village, IL, USA).

2.5 Glucagon secretion and content measurements.

Mice were euthanized by cervical dislocation, and islets were isolated by collagenase digestion as previously described [18]. Freshly isolated islets from non-pregnant controls and G18.5 pregnant mice were left to recover for 2 hours at 37ºC and 5% CO_2 in the isolation medium, which contained (in mM): 115 NaCl, 5 KCl, 10 $NaHCO_3$, 1.1 $MgCl_2$, 1.2 NaH_2CO_4, 2.5 $CaCl_2$, 25 HEPES, 5 glucose and 0.25% BSA (pH 7.4). Groups of 15 islets were preincubated for 1 hour in 500 µl of medium with 0.5 mM glucose (G), 0.1% BSA and the following composition (in mM): 140 NaCl, 4.5 KCl, 1 $MgCl_2$ and 20 HEPES (pH 7.4). Islets were then exposed for 1 hour to 300 µl of medium with the same composition and different stimuli: 0.5 mM G, 11 mM G, or 0.5 mM G plus 10 nM insulin. Finally, media were collected and glucagon concentrations were measured by ELISA (Mercodia AB, Uppsala, Sweden). To determine glucagon content, islets were collected and incubated overnight at 4°C in 20 µl of a lysis buffer

(75% ETOH, 24,6% dH20 and 0,4% of 30% HCl) and then centrifuged at 14.000 rpm for 4 minutes. Glucagon content was measured in the supernatant by ELISA. Total protein concentration was analyzed using the Bradford dye method [18].

Measurement of glucagon secretion from α-TC1.9 cells was performed with the media described for experiments with isolated islets. On day 8 after hormonal treatment, cells were preincubated for 2 hours with 500 µl of secretion media with 5.6 mM G and then, incubated for 1 hour with the appropriate stimuli: 0.5 mM G, 11 mM G or 0.5 mM G plus 10 nM insulin. Next, 400 µl were collected and used to measure glucagon secretion by ELISA. To determine glucagon content, cells were recovered from the wells and treated with 200 µl of lysis buffer overnight at 4ºC. To measure glucagon release or content, aprotinin (20 mg/l) was included in all media [18].

2.6 RNA isolation and real time PCR.

After 8 days of hormonal treatment, total RNA from α-TC1.9 cells was extracted using the RNeasy Mini kit (Qiagen, Madrid, Spain) and quantified by optical density at 260 and 280 nm using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA synthesis was carried out from 0.5 µg of total RNA using the High Capacity cDNA Reverse Transcription RNA kit (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10’ at 25ºC, 120’ at 37ºC and 5’ at 85ºC, cooling down the samples at 4ºC after these steps. Quantitative PCR assays were performed using a CFX96 Real Time System (Bio-Rad, Hercules, CA, USA) with 1µl of cDNA in a total final volume of 10 µl, containing 200 nM of each primer and 1X IQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA). Samples were subjected to the following conditions: 3 min at 95°C, 45 cycles of 5 s at 95°C, 5 s at 60°C and 10 s at 72°C, and a melting curve of 65 to 95°C with a slope of 0.1°C/s.

213 The housekeeping gene HPRT (Hypoxanthine-guanine phosphoribosyl transferase) was
214 used as the endogenous control for quantification. The resulting values were expressed
215 as relative expression compared with control levels ($2^{-\Delta\Delta Ct}$) [20]. Further information
216 about the primers sequences can be found in the Supplementary Table 1.
217

218 **2.6 Statistical analysis.**

219 Statistical analysis was performed with GraphPad Prism 7.0 software (GraphPad
220 Software Inc., San Diego, CA, USA). Data was shown as the mean ± SEM. Student's t
221 test, one-way ANOVA or two-way ANOVA were applied according to the set of groups
222 that were compared. Non-parametric tests were performed when data did not meet the
223 assumption of normality. Except when indicated, a Dunnett's post hoc test or a
224 Bonferroni post hoc test was performed after one-way ANOVA or two-way ANOVA
225 analysis, respectively. Results were considered significant at $p < 0.05$.
226
227

3. Results.

3.1 Pregnant mice exhibit increased pancreatic alpha-cell mass due to hypertrophy and hyperplasia on gestational day G18.5.

To study the morphological characteristics of the pancreatic alpha-cell during pregnancy, we first analyzed the alpha-cell population at different time points during mouse gestation: G12.5, G15.5 and G18.5. Major beta-cell morphological changes have been reported at these time periods [4,21]. The alpha-cell area and mass were significantly increased on G18.5 (Fig. 1A, B). Additionally, the alpha-cell size was augmented on G18.5 and there was a high tendency for increased alpha-cell number per islet compared with controls (Fig. 1C, D). These morphological changes suggested that hypertrophy and, probably, hyperplasia were part of the regulatory events involved in the alpha-cell mass expansion observed on G18.5. This alpha-cell mass increase occurred with a similar temporal pattern as that described for the expansion of the pancreatic beta-cell during mouse pregnancy, which peaks around G16-G18.5 [22,23]. Given that different morphological parameters were significantly altered on G18.5, we focused on this time point in the following experiments.

3.2 Alpha-cell mass expansion during late pregnancy is mainly due to increased alpha-cell proliferation.

Although it has been claimed that pancreatic beta-cell mass expansion during gestation is essentially driven by enhanced proliferation, other processes like apoptosis, neogenesis and transdifferentiation have also been proposed [1,2,4]. To determine the processes responsible for the pancreatic alpha-cell mass expansion, we first analyzed proliferation by measuring Ki-67 antigen expression in glucagon-positive cells [19]. A two-fold increase in alpha-cell proliferation was observed on G18.5 (Fig. 1E).

Additionally, the apoptotic rate was found to be very low in pancreatic alpha-cells and no significant differences were observed on G18.5 compared with controls (Fig. 1F).

Given that few studies have suggested that beta-cell neogenesis from ductal cells may also play a role during pregnancy in humans [24] and mice [21,25,26], we explored whether this process was also contributing to alpha-cell growth during gestation. The analysis of the presence of glucagon-containing cells co-stained with the ductal marker pan-cytokeratin (PanCK) [19] revealed that their occurrence was low and not different between both groups (Fig. 2A, B). In order to determine whether islet-cell transdifferentiation may also occur during gestation as part of the pancreas adaptive response, we performed an immunofluorescence analysis to quantify the presence of glucagon and insulin double-positive cells [27,28]. We observed 4 double-positive cells out of 1602 total alpha-cells in controls (n=3) and 6 double-positive cells out of 1815 alpha-cells in pregnant mice (n=3) (Fig. 2C), suggesting a negligible involvement of alpha-beta reprogramming. Overall, these results indicate that alpha-cell proliferation is probably the main contributor to the increased alpha-cell mass during late pregnancy.

3.3 Pregnant mice on G18.5 exhibit hypoglucagonaemia and impaired glucagon secretion.

When plasma parameters associated with glucose homeostasis were analyzed, we found a decrease in glycaemia in pregnant mice on G18.5 compared with controls (Fig. 3A). Pregnant animals also exhibited hypoglucagonaemia (Fig. 3B), while plasma insulin levels showed a non-significant increase (Fig. 3C). To evaluate alpha-cell functional activity, we performed a static glucagon secretion experiment using freshly isolated islets. Islets were challenged with stimulatory (0.5 mM) or inhibitory (11 mM) glucose (G) concentrations for the pancreatic alpha-cell [29] and also with insulin,

278 which reduces glucagon release [30]. As expected, islets from non-pregnant mice
279 exhibited vigorous glucagon secretion at 0.5 mM G, while hormonal release was
280 significantly inhibited in the presence of 11 mM G or 10 nM insulin (Fig. 3D),
281 consistent with prior studies [29,30]. Remarkably, glucagon secretion at 0.5 mM G was
282 significantly reduced in islets from pregnant mice compared to controls (Fig. 3D and
283 Supplementary Fig. 1 for secretion normalized to glucagon islet content and total islet
284 protein, respectively). Glucagon content was decreased in pancreatic islets from
285 pregnant animals compared with controls, although this only reached statistical
286 significance in the insulin condition (Fig. 3E).
287
288 **3.4 Lactogenic hormones and estradiol affect proliferation, glucagon release and**
289 **proglucagon mRNA expression in α-TC1.9 cells.**
290 PL, PRL, P and E_2 hormones promote different aspects of the adaptive response
291 of the pancreatic beta-cell during pregnancy [3]. To assess whether these hormones also
292 function to regulate the alpha-cell changes described above, we analyzed proliferation
293 and glucagon release from α-TC1.9 cells. These cells were treated for 8 days with
294 hormone concentrations within the range described in pregnant mice [3,31-34] and in
295 similar *in vitro* studies [32,35]. While PL and PRL stimulated proliferation, E_2 induced
296 the opposite effect (Fig. 4).
297 Based on the results obtained from the *ex vivo* glucagon secretion studies (Fig.
298 3), we also examined the potential role of pregnancy hormones in glucagon release from
299 α-TC1.9 cells. After 8 days of hormone exposure [32], glucagon secretion was analyzed
300 after incubation of cells with 0.5 mM G, 11 mM G or 0.5 mM G plus 10 nM insulin for
301 1 hour. Similar to isolated islets (Fig. 3), α-TC1.9 cells treated with vehicle exhibited
302 maximal glucagon secretion at 0.5 mM G and significant decreases at both 11 mM G

303 and 0.5 mM G plus insulin. Interestingly, glucagon release at 0.5 mM G was
304 significantly inhibited in the cells treated with PL, PRL and E_2 (Fig 5A). Similar results
305 were obtained when glucagon release was normalized to islet protein content
306 (Supplementary Fig. 2A). No differences were found between groups in the glucagon
307 content or in glucagon gene expression, with the exception of a decrease in glucagon
308 mRNA after E_2 exposure (Fig. 5B, C and Supplementary Fig. 2B, C). Overall, our
309 findings in both pancreatic alpha-cells and α-TC1.9 cells indicate that the gestational
310 hormones PRL, PL and E_2 are likely involved in the alpha-cell alterations during
311 pregnancy.
312
313

4. Discussion.

During pregnancy, the mother undergoes major hormonal and metabolic changes to meet the energy requirements of the growing fetus. These changes involve a maternal decrease in peripheral insulin sensitivity to ensure the glucose gradient for the fetus. To maintain glucose homeostasis in this situation, important morphofunctional adaptations take place in the pancreatic beta-cell to increase hormonal secretion and compensate for the insulin resistance [1,2,4,36]. A deficient pancreatic beta-cell adaptation can lead to impaired glucose homeostasis and GDM [1,2,5]. It has been described that pregnancy hormones play a key role in the development of both insulin resistance and beta-cell adaptations during gestation [3], yet there is little information about the alterations of the pancreatic alpha-cell during pregnancy and the potential function of gestational hormones.

In the present study, we analyzed the morphofunctional features of pancreatic alpha-cells during pregnancy. We found a significant increase in alpha-cell mass at G18.5, which was mainly associated with hypertrophy and hyperplasia, a situation similar to that reported in beta-cells [4,22]. These findings are in accordance with a previous study showing alpha-cell expansion during pregnancy in a similar period [22]. The increase in alpha-cell mass on G18.5 was not associated with changes in apoptosis, which remained at low levels, similar to what has been reported for beta-cells during pregnancy [24,37]. This result was not unexpected, since alpha-cell apoptosis is very low in non-pregnant mice [38] and this islet cell type exhibits a high resistance to pro-apoptotic stimuli compared with beta-cells [39].

Although proliferation seems to be the major contributor to beta-cell mass growth in rodent pregnancy, few studies have observed that up to 10-25% of beta-cells may come from other non-beta-cell sources [2,25,26]. In this regard, neogenesis from

ductal cells has been postulated as an important contributor to beta-cell mass during human pregnancy [24]. However, since other studies failed to detect beta-cell neogenesis [40,41], this issue remains controversial. Of note, we found a low proportion of glucagon-containing cells expressing the ductal marker PanCK that did not change between control and pregnant animals. Thus, these findings indicate that pancreatic ductal cells do not act as an alpha-cell source during pregnancy. Transdifferentiation among different islet cell types has been reported under some pathophysiological conditions and with genetically modified animal models [27,28]. In our study, we observed a similar very low number of islet cells expressing both insulin and glucagon in control and pregnant mice, which suggests that transdifferentiation programs may not have a significant impact on alpha-cell mass during pregnancy.

Although E_2 and P may also have an effect on beta-cell proliferation [32,42], PL and PRL have been described as key regulators of gestational beta-cell replication [1,22,23,37]. Treatment of α-TC1.9 cells with PL and PRL increased proliferation, suggesting an important role for these hormones, which is similar to findings in beta-cells [32]. The PRL receptor has been reported in neonatal rat pancreatic alpha-cells [43]. However, while PRL activated the JAK/STAT5 pathway in rat beta-cells, this effect was not observed in alpha-cells [35]. This suggests that PRL may activate STAT-independent signaling routes in glucagon-secreting cells, as has been reported in other cell types [44]. Unlike PL and PRL, E_2 diminished α-TC1.9 cell proliferation. Progesterone also produced a similar trend, as expected since this hormone counteracts PRL-induced rat beta-cell proliferation [32]. The role of E_2 in beta-cell proliferation is still unclear: while this hormone partially reverses PRL-induced BrdU incorporation in rat islets [32], it has been shown that E_2 increases proliferation in insulinoma INS1 cells and dispersed rat beta-cells after 48 hours of culture [42]. Given that these studies were

performed with pharmacological doses, it is difficult to draw conclusions to the physiological context. Thus, although E_2 proliferative effects on the beta-cell may differ depending on experimental conditions, our findings indicate that E_2 may decrease alpha-cell replication. Overall, all these results indicate that PRL and PL may have a prominent function in the regulation of the alpha-cell mass expansion during pregnancy, as has been reported for the pancreatic beta-cell [2,4,32].

It is well known that plasma insulin levels and glucose-stimulated insulin secretion (GSIS) from pancreatic beta-cells follow a dynamic pattern during pregnancy [1,2,32,36,45,46]. One of the most important adaptive changes in pregnancy is a lowering of the threshold for GSIS [45,46]. This threshold changes throughout pregnancy peaking at G15, when islets release 8-fold more insulin at 5.6 mM glucose. At G19, this effect is diminished to 4-fold increase in insulin secretion, and reaches control levels at G20 [45,46]. Additionally, it has been reported in mice that whole body insulin sensitivity both is higher at G19 compared with G16 and is similar to non-pregnant animals [47]. This insulin sensitivity level at G19 near control conditions [47], together with a decreased threshold for GSIS at this gestational day [45], may explain the lower glucose levels and the trend to high insulin concentrations found in the present work. A similar scenario has been previously described in late pregnancy in mice [5]. Likewise, higher insulinemia at mid gestation compared with late pregnancy and non-pregnant controls has also been described in mice [21]. This situation should be considered as an aspect of late pregnancy reconditioning to prepare the mother for labor and lactation [46].

Contrary to the situation of beta-cells, there is nearly no evidence about glucagon secretion from pancreatic alpha-cells during pregnancy. In agreement with a previous study [12], we observed that pregnant mice exhibited hypoglucagonemia on

G18.5. In women, plasma glucagon concentrations rise during the first and second trimesters but they decrease at late pregnancy [11]. The hypoglucagonemia observed in pregnant animals was further supported by *in vitro* glucagon release experiments showing that alpha-cell secretion from freshly isolated islets was down-regulated in pregnant mice. Additionally, glucagon content also seemed to be reduced in the islets from pregnant female. Because paracrine influences should not affect alpha-cells at 0.5 mM glucose [6,7,29], the reduced glucagon secretion may reflect a direct action on these cells, probably a down-regulation at the level of glucose-sensing, signal transduction and/or exocytosis. In pregnant rats, basal glucagon release from isolated islets was also found to be reduced at 2.5 mM glucose [48]. The lower plasma glucagon levels found during late gestation could be the result of a direct regulation by pregnancy-induced factors and/or by paracrine inhibitory signals acting on alpha-cells, like insulin or serotonin [12,30,49], whose intra-islet secretion from beta-cells is increased during gestation [1,50]. In the present work, the *in vitro* experiments with α-TC1.9 cells also point to a direct role for gestational hormones in late pregnancy hypoglucagonemia. PL, PRL and E_2 all diminished glucagon secretion at 0.5 mM G. A previous study using purified primary alpha-cells also showed that exposure to 10 nM E_2 for 48 hours down-regulated glucagon content and release [51]. In line with this, E_2 produced an inhibitory effect on α-TC1.9 cells at the level of secretion and glucagon mRNA expression in our experiments. To the best of our knowledge, there are no previous data describing PL and PRL actions on alpha-cell secretion. Overall, these results indicate that PL, PRL and E_2 are likely involved in the inhibitory effect observed in glucagon secretion from isolated islets in pregnant mice. In any case, given the complexity of the signals that regulate the adaptations of the endocrine pancreas during gestation [36], we cannot rule out the involvement of other hormones.

It has been reported that genetic ablation of either prohormone convertase 2 or glucagon leads to alpha-cell hyperplasia and hypertrophy [52-54]. These results have been interpreted as a compensatory response to the limited hepatic glucagon signaling associated with the lower plasma glucagon levels in these animals [54]. Thus, it is plausible that the alpha-cell hyperplasia and hypertrophy described here in pregnant mice could be derived from their hypoglucagonemia. Recent findings have also shown that interrupted glucagon receptor signaling can induce hepatic amino-acid release and hyperaminoacidemia, which seems to be linked to mTOR-mediated alpha-cell proliferation [55]. Further studies will be required to better understand the whole scenario.

In addition to the potential *in vivo* interactions described above that may take place during pregnancy, the results obtained with the alpha-cell line also point to a direct role of PL and PRL in the dual actions on alpha-cells during gestation. Activation of the PRL receptor involves the stimulation of at least three main signaling pathways in the pancreatic beta-cell and other cell types: JAK2/STAT5, MAPK and PI3K [1,44]. In the case of mouse alpha-cells and alpha-TC1 cells, activation of the PI3K pathway has been associated with both the inhibition of glucagon secretion [6,30] and the increase of proliferation [56]. Thus, it is plausible that PRL receptor activation may produce opposite actions on alpha-cell proliferation and secretion through PI3K signaling. A similar situation has also been described for insulin via PI3K [6,7,30,56] and for glucagon-like peptide 1 (GLP-1) via the PKA signaling pathway [6,7,57,58].

In any case, we should take with some caution the PRL and PL effects observed in our *in vitro* model, when compared with *in vivo* conditions. The control of pancreatic alpha-cell secretion is a complex process compared with that of the beta-cell [7,59,60]. It has been shown that, in addition to glucagon, pancreatic alpha-cells can also secrete

other signaling molecules like GLP-1, acetylcholine, GIP (gastric inhibitory polypeptide) and glutamate, which can subsequently affect alpha-cell function by autocrine regulation [6,7,59,60]. These processes can be induced under certain conditions, and usually involve long-term stimulation (like chronic *in vitro* conditions) rather than short-term acute modulation. Additionally, several stimuli like glucose or adrenaline have been shown to produce a dose-dependent biphasic action on glucagon release [57,60], which has been attributed to the particular electrical activity and ATP-dependent K^+ channel activity pattern that specifically regulates pancreatic alpha-cell secretion [57,60]. Thus, we cannot totally discard that, in other conditions, PRL and PL may induce alternative secretory effects on pancreatic alpha-cells during pregnancy, which represents a complex scenario with multiple signals.

In summary, the present findings show that the pancreatic alpha-cell undergoes important morphofunctional changes during pregnancy. These changes are likely regulated by the main pregnancy hormones that have been implicated in the adaptive response of the pancreatic beta-cell during this physiological condition. These alpha-cell adaptations during an insulin-resistant condition might be crucial for the maintenance of an adequate glucose and metabolic milieu for the mother and the fetus. Indeed, the key role of glucagon during pregnancy has been previously pointed out by studies showing that interruption of glucagon signaling results in fetoplacental defects and alterations in the fetal metabolic environment [10], and that this hormone regulates the metabolism of placental glycogen cells [8, 9]. Therefore, the attenuated alpha-cell function on G18.5 described here could be an adaptation to prevent a potential evolution to a hyperglycemic state. Indeed, since GDM has been associated with several alterations in the function of these islet cells and glucagon hypersecretion [13-15], an inadequate pancreatic alpha-cell adaptation to pregnancy may promote a hyperglycemic condition.

Our current findings provide novel information about the complex adaptations of the endocrine pancreas during pregnancy to have a more complete view of the general scenario. These data would be also interesting for the design of new therapeutic strategies in GDM.

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Declarations of interests.

The authors have no interests to declare.

Author contributions.

I.Q. and A.N. designed the experiments; C.Q.C. and E.T. performed and analyzed the experiments; C.Q.C, E.T., P.A.M., L.M., I.Q. and A.N. interpreted the results; C.Q.C., I.Q. and A.N. wrote the manuscript. All authors contributed to the discussion, reviewed the manuscript and approved the final version of the article.

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635 glucagon release by differential modulation of N- and L-type Ca2+ channel-dependent
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643 glucose: paracrine, intrinsic or both? *Diabetes Obes Metab* 2011;13 Suppl 1:95-105.

644

645

646 **Figure legends**

647 **Figure 1.** Increase in alpha-cell mass, area and cell size in pregnant mice on G18.5. **(A-**
648 **D)** Different morphological parameters were measured on gestational days G12.5,
649 G15.5 and G18.5 in pregnant mice and non-pregnant controls. **(A)** Alpha-cell area (n=5
650 mice per group). **(B)** Alpha-cell mass (n=5 mice per group). **(C)** Alpha-cell number per
651 islet (100 islets were randomly selected from 5 mice per group). **(D)** Alpha-cell size
652 (100 islets were randomly selected from 5 mice per group). **(E)** Alpha-cell proliferation
653 (%) in non-pregnant (control) and pregnant mice on G18.5 (n=4 controls; n=5 pregnant
654 mice). **(C)** Alpha-cell apoptosis (%) analyzed by TUNEL in control and pregnant mice
655 (n=5 mice each group). Values represent mean ± SEM. *p<0.05. One way-ANOVA
656 followed by Dunnet's post hoc test (A, B); One way-ANOVA followed by Fisher's
657 Least Significant Difference (LSD) test (C); Kruskal-Wallis followed by Dunn's post
658 hoc test (D). Mann-Whitney test (E); Student's t test (F).
659

660 **Figure 2.** Ductal glucagon-containing and insulin-glucagon double-positive cells are not
661 increased in pregnant mice on G18.5. **(A)** Percentage of double-positive cells for
662 glucagon and PanCK in control and pregnant mice on G18.5 (n=5 mice each group). **(B)**
663 Representative images showing the cellular staining for glucagon (red), PanCk (green)
664 and the nuclear labeling with Hoechst (blue) in pancreatic sections from controls and
665 pregnant mice. Boxed areas are enlarged on the right. White arrows indicate double-
666 positive cells. **(C)** Representative images showing the cellular staining for glucagon
667 (red), insulin (green) and the nuclear labeling with Hoechst (grey) in pancreatic sections
668 from controls and pregnant mice. Boxed areas are enlarged on the right. White arrows
669 indicate double-positive cells. Values represent mean ± SEM. *p<0.05. Student's t test
670 (A).

671

672 **Figure 3.** Plasma parameters and *ex vivo* glucagon secretion on G18.5. **(A)** Glycaemia
673 in control and pregnant mice (n=16 and n=8 animals, respectively). **(B)** Glucagon
674 plasma levels in control and pregnant mice (n=11 and n=9 animals, respectively). **(C)**
675 Insulin plasma levels in control and pregnant mice (n=12 and n=8 animals,
676 respectively). All plasma parameters were measured in non-fasted state conditions **(D)**
677 Glucagon secretion normalized by content from freshly isolated islets of controls and
678 pregnant mice at 0.5 mM glucose (G), 11 mM G and 0.5 mM glucose plus 10 nM
679 insulin (INS) (n=18-19 control mice; n=11 pregnant mice; 15 islets per animal and
680 condition were used). **(E)** Glucagon content in each condition in non-pregnant mice and
681 pregnant G18.5 mice (n=19-20 control mice; n=12 pregnant mice; 15 islets per animal
682 and condition were used). Values represent mean ± SEM. *p<0.05; **p<0.01;
683 ****p<0.001. Student's t test (A-C); Two-way ANOVA followed by Bonferroni's post
684 hoc test (D-E).

685

686 **Figure 4.** Effects of the pregnancy hormones P, PL, PRL and E_2 on the proliferation of
687 α-TC1.9 cells. **(A)** Quantification of BrdU-positive cells relative to control conditions
688 (vehicle) in α-TC1.9 cells treated for 8 days with PL (500 ng/ml), PRL (500 ng/ml), P
689 (100 ng/ml) and E_2 (100 pmol/l). Three different experiments were performed (n= 8
690 coverslips per condition; at least 2.000 cells were counted per coverslip). **(B)**
691 Representative images showing nuclei stained for propidium iodide (red) and BrdU
692 (green). Values represent mean ± SEM. *p<0.05. One way-ANOVA followed by
693 Fisher's Least Significant Difference (LSD) test (A).

694

695 **Figure 5.** Effect of pregnancy hormones on glucagon secretion, content and
696 proglucagon mRNA expression in α-TC1.9 cells. Cells were treated for 8 days with PL
697 (500 ng/ml), PRL (500 ng/ml), P (100 ng/ml) and E_2 (100 pmol/l). **(A)** Glucagon release
698 from α-TC1.9 cells normalized by glucagon content was measured after 1 hour
699 incubation in 0.5 mM G, 11 mM G or 0.5 mM G plus 10 nM insulin. **(B)** Glucagon
700 content normalized by total protein from α-TC1.9 cells exposed at 0.5 mM glucose in A.
701 **(C)** Proglucagon mRNA expression relative to the control (vehicle). Three different
702 experiments were performed: n=7-9 wells per condition in (A), n=8-9 wells per
703 condition in (B), and n=6 wells per condition in (C). Values represent mean ± SEM.
704 Two-way ANOVA followed by Bonferroni's post hoc test was performed in (A), where
705 letters indicate $p<0.05$: a, 11 mM G and 0.5 mM G + 10 nM INS versus 0.5 mM G in
706 vehicle experiments; b, 0.5 mM G in hormone-treated conditions versus 0.5 mM G in
707 vehicle; c, 11 mM G in PRL conditions versus 11 mM G vehicle. One-way ANOVA
708 followed by Dunnet's post hoc test was performed in (B) and (C), *$p<0.05$.
709

Highlights

- Pregnancy promotes pancreatic alpha-cell mass expansion.
- Alpha-cell proliferation and size are increased during late pregnancy.
- Hypoglucagonemia and impaired glucagon secretion are present during late pregnancy.
- Pregnancy hormones are potentially involved in these alpha-cell alterations.

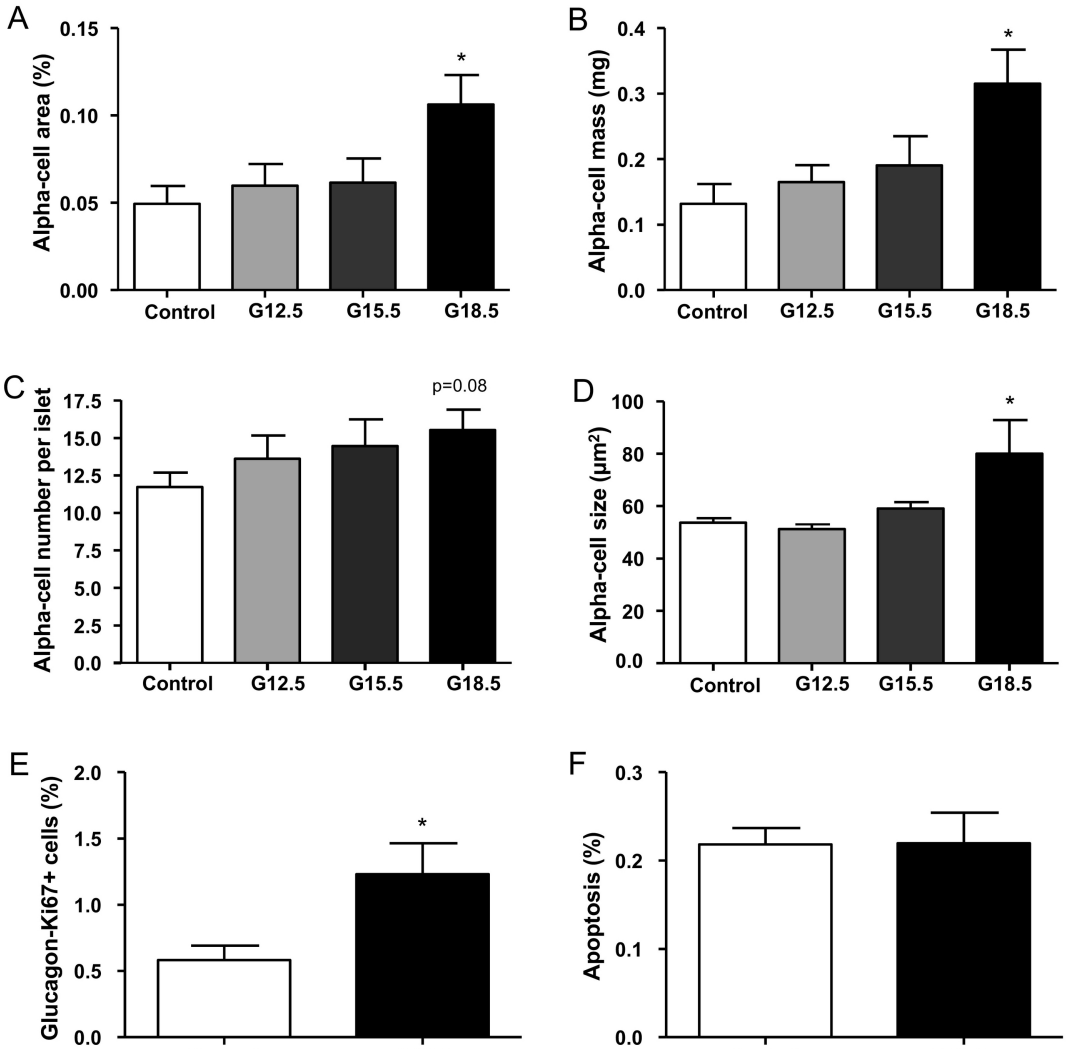
A
Alpha-cell area (%)
0.15
0.10
0.05
0.00
*
Control
G12.5
G15.5
G18.5
B
Alpha-cell mass (mg)
0.4
0.3
0.2
0.1
0.0
*
Control
G12.5
G15.5
G18.5
C
Alpha-cell number per islet
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
p=0.08
Control
G12.5
G15.5
G18.5
D
Alpha-cell size (µm²)
100
80
60
40
20
0.0
*
Control
G12.5
G15.5
G18.5
E
Glucagon-Ki67+ cells (%)
2.0
1.5
1.0
0.5
0.0
*
Control
G18.5
F
Apoptosis (%)
0.3
0.2
0.1
0.0
Control
G18.5

Figure 1

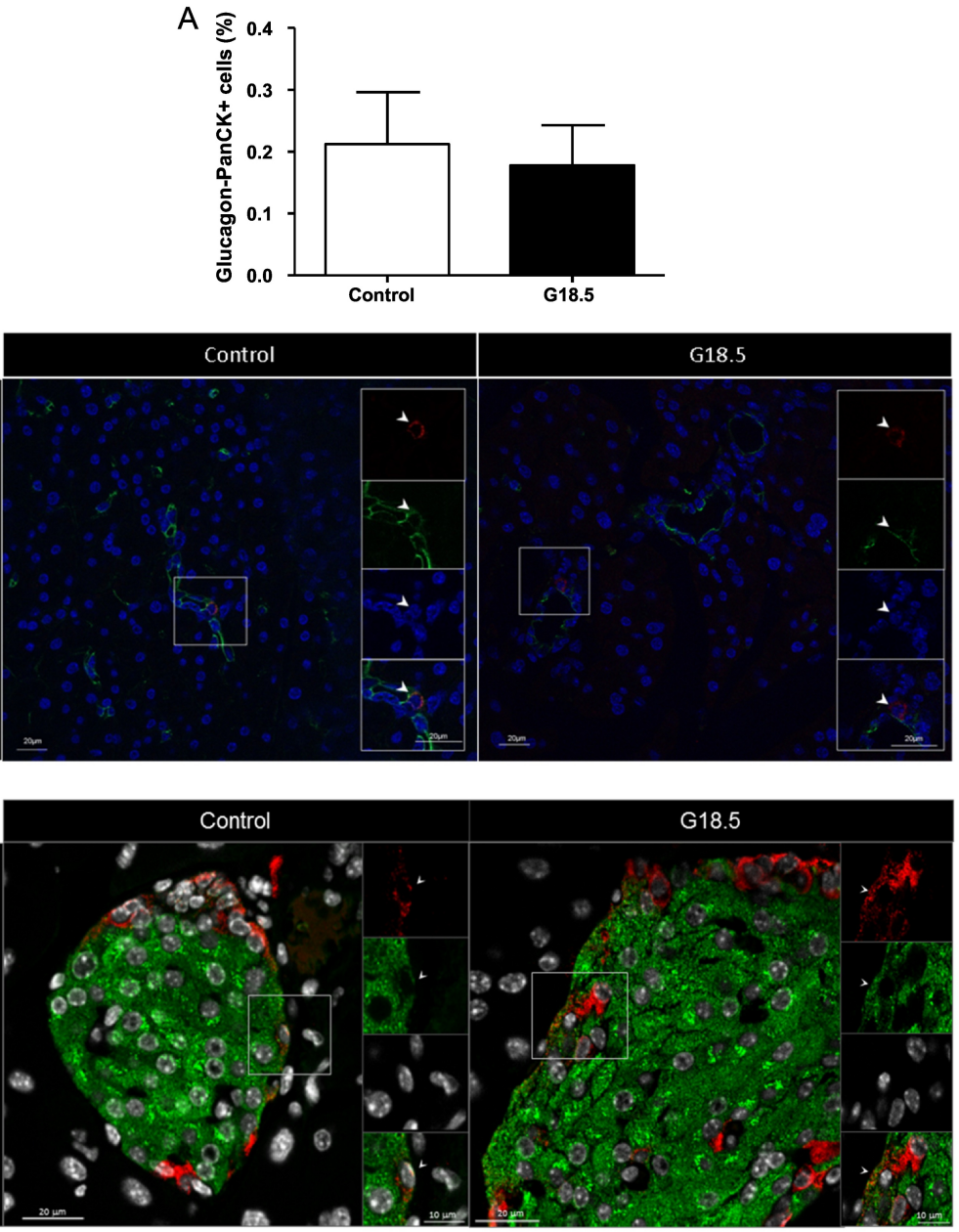
A
Glucagon-PanCK+ cells (%)
0.4
0.3
0.2
0.1
0.0
Control
G18.5
B
Control
G18.5
Glucagon/TUNEL/Hoechst
20µm
C
Control
G18.5
Glucagon/Insulin/Hoechst
20 µm
10 µm

Figure 2

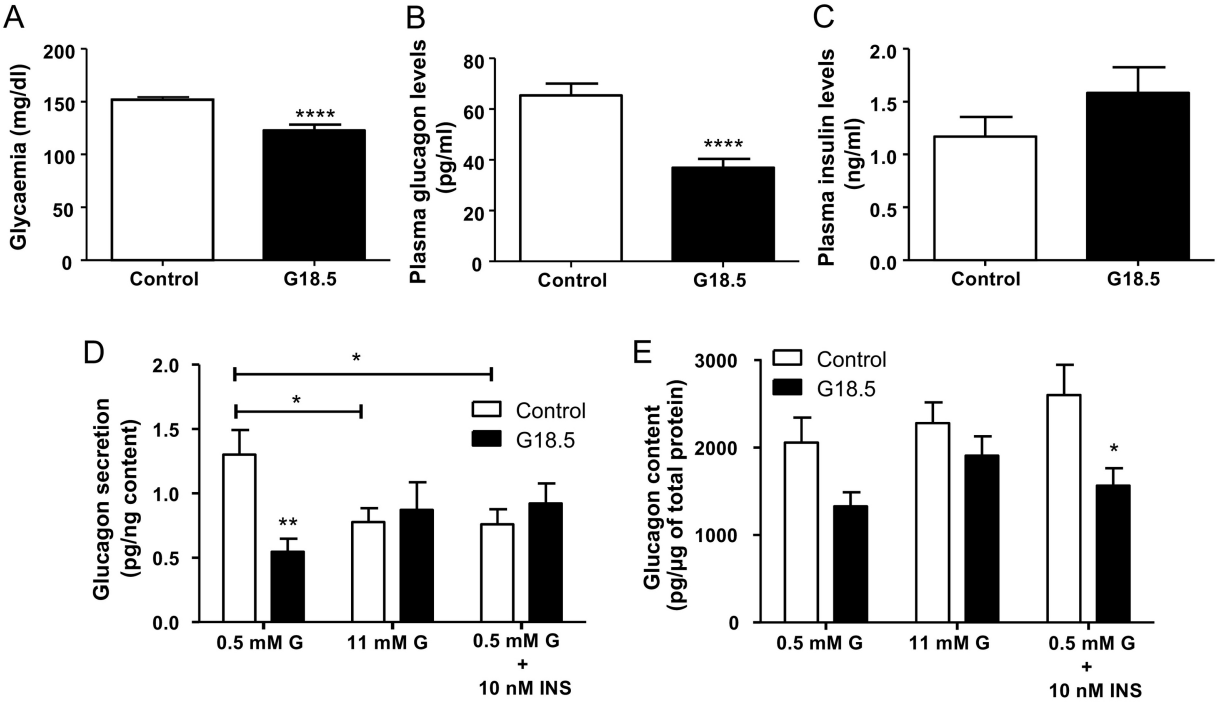
A
Glycaemia (mg/dl)
200
150
100
50
0
****
Control
G18.5
B
Plasma glucagon levels (pg/ml)
80
60
40
20
0
****
Control
G18.5
C
Plasma insulin levels (ng/ml)
2.0
1.5
1.0
0.5
0.0
Control
G18.5
D
Glucagon secretion (pg/ng content)
2.0
1.5
1.0
0.5
0.0
*
*
**
Control
G18.5
0.5 mM G
11 mM G
0.5 mM G + 10 nM INS
E
Glucagon content (pg/µg of total protein)
3000
2000
1000
0
Control
G18.5
*
0.5 mM G
11 mM G
0.5 mM G + 10 nM INS

Figure 3

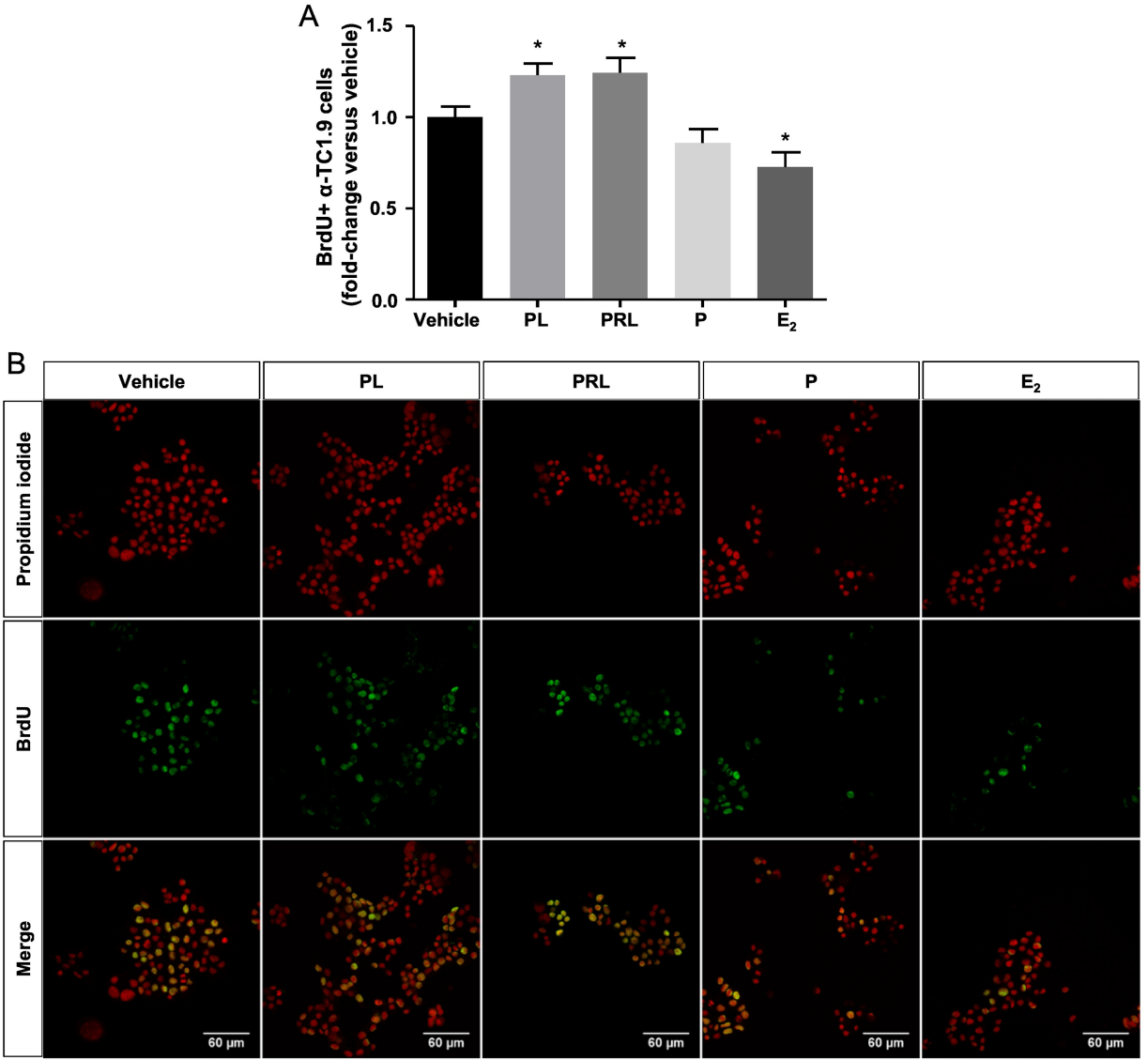
A
BrdU+ α-TC1.9 cells
(fold-change versus vehicle)
1.5
1.0
0.5
0.0
*
*
*
Vehicle
PL
PRL
P
E2
B
Vehicle
PL
PRL
P
E2
Propidium iodide
BrdU
Merge
60 µm
60 µm
60 µm
60 µm
60 µm

Figure 4

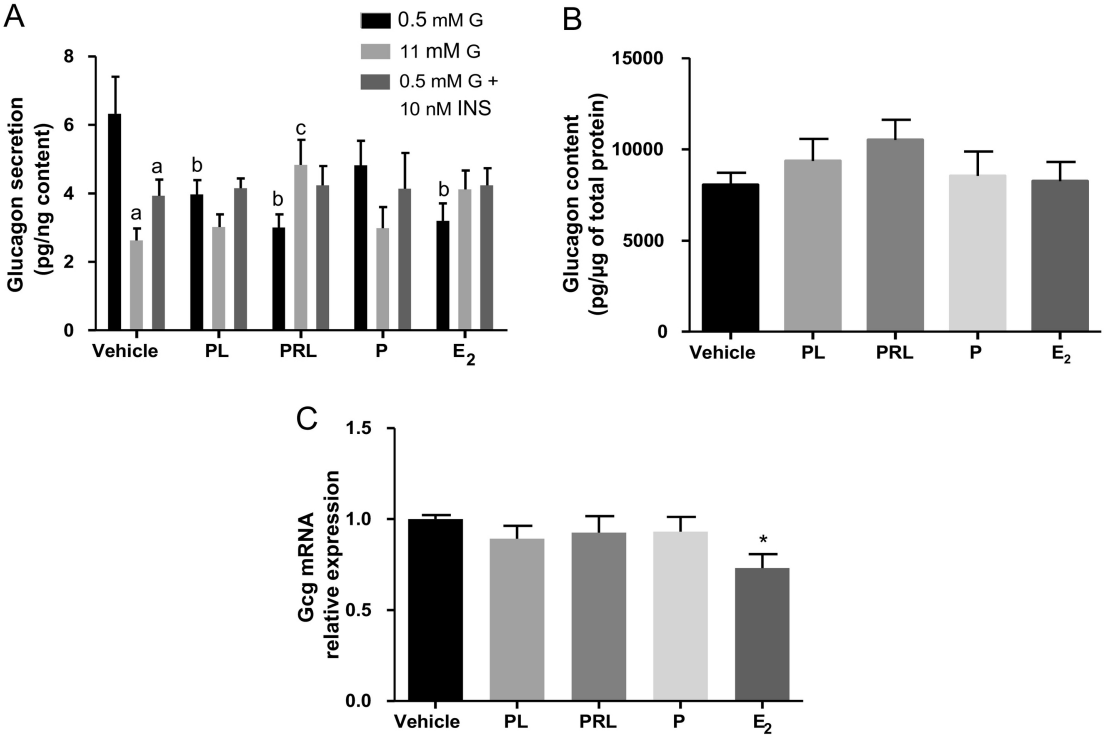
A
0.5 mM G
11 mM G
0.5 mM G +
10 nM INS
Glucagon secretion
(pg/ng content)
8
6
4
2
0
a
a
b
b
c
b
a
b
Vehicle
PL
PRL
P
E2
B
Glucagon content
(pg/µg of total protein)
15000
10000
5000
0
Vehicle
PL
PRL
P
E2
C
Gcg mRNA
relative expression
1.5
1.0
0.5
0.0
*
Vehicle
PL
PRL
P
E2

Figure 5