

# Intraislet production of GLP-1 by activation of prohormone convertase 1/3 in pancreatic $\alpha$ -cells in mouse models of $\beta$ -cell regeneration

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**Key words:** pancreatic  $\beta$ -cells,  $\alpha$ -cells, islets, glucagon, glucagon-like peptide-1, prohormone processing, neonatal development, obesity, pregnancy, diabetes

**Abbreviation:** GLP, glucagon-like peptide; PC, prohormone convertase

The islet of Langerhans is a highly vascularized micro-organ consisting of not only  $\beta$ -cells but multiple cell types such as  $\alpha$ -, delta-, pancreatic polypeptide- and epsilon-cells that work together to regulate glucose homeostasis. We have recently proposed a new model of the neonatal islet formation in mice by a process of fission following contiguous endocrine cell proliferation in the form of branched cord-like structures in embryos and newborns. There exist large stretches of interconnected islet structures along large blood vessels in the neonatal pancreas, which, upon further development, segregate into smaller fragments (i.e., islets) that eventually become more spherical by internal proliferation as seen in the adult pancreas.  $\alpha$ -cells span these elongated islet-like structures in the developing pancreas, which we hypothesize represent sites of fission and facilitate the eventual formation of discrete islets. The  $\alpha$ -cells express both prohormone convertase 2 and 1/3 (PC2 and PC1/3, respectively), which resulted in the processing of the proglucagon precursor into glucagon-like peptide 1, thereby leading to local production of this important  $\beta$ -cell growth factor. Furthermore, while  $\alpha$ -cells in the adult basically only express PC2, significant activation of PC1/3 is also observed in mouse models of insulin resistance such as pregnant, *ob/ob*, *db/db* and prediabetic NOD mice, which may be a common mechanism in proliferating  $\beta$ -cells. Our study suggests an important role of  $\alpha$ -cells for  $\beta$ -cell proliferation and further for the endocrine cell network within an islet.

## Introduction

Insulin-secreting  $\beta$ -cells play a pivotal role in maintaining glucose homeostasis and in the pathogenesis of diabetes mellitus. However, the  $\beta$ -cells function within a micro-organ composed of multiple cell types, including glucagon-secreting  $\alpha$ -cells, somatostatin-secreting  $\delta$ -cells, pancreatic polypeptide-secreting PP cells and ghrelin-secreting  $\epsilon$ -cells, and are also enriched with a dense vasculature network.

Islets demonstrate a striking plasticity that is observed among different species, within the same species and under physiological and pathological conditions.<sup>1,2</sup> Changes in cellular composition, such as  $\beta$ - to  $\alpha$ -cell ratio, are often accompanied with more central localization of  $\alpha$ -cells. Islet formation occurs by a process of fission following contiguous endocrine cell proliferation in the form of branched cord-like structures in embryos and newborns.<sup>3</sup> Extended stretches of interconnected islet structures are present along large blood vessels in the neonatal pancreas. Upon further development, the interconnected islets segregate into smaller fragments (i.e., islets), which eventually become more spherical by internal proliferation, as seen in the adult pancreas. Endocrine-

cell cluster formation was also seen in a teratoma model of ES cells transplanted under the kidney capsule.<sup>4</sup> These studies imply that  $\beta$ -cell differentiation, proliferation and regeneration occur within the context of the endocrine cell network.

In this study, we focused on the possible role of  $\alpha$ -cells in  $\beta$ -cell proliferation and regeneration. Here we report the important role of temporally regulated expression of PC1/3 in  $\alpha$ -cells. Activation of PC1/3 leads to the processing of the major proglucagon fragment that results in the local production of GLP-1, which has been considered to occur only in intestinal L-cells in adults.<sup>5</sup> Elucidating intrinsic signaling pathways in the intraislet endocrine cell network may lead to the identification of novel therapeutic targets.

## Results

**$\alpha$ -cell hyperplasia and activation of PC1/3 expression in  $\alpha$ -cells.** Activation of PC1/3 expression in  $\alpha$ -cells was observed in neonates, prediabetic NOD mice, pregnant mice, *ob/ob* mice (leptin deficiency) and *db/db* mice (leptin receptor deficiency). Representative immunohistochemical analysis is shown in

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**Figure 1A.** Note that  $\beta$ -cells in all specimens examined expressed PC1/3, which is a major enzyme in the processing of proinsulin to insulin and C-peptide.<sup>6</sup> Though the majority of  $\alpha$ -cells in adult wild-type mice at 6-mo of age do not express PC1/3, some  $\alpha$ -cells were positive for PC1/3 staining (Fig. 1B-WT).

Changes in the  $\alpha$ -cell to  $\beta$ -cell ratio were examined in the developing pancreas (E17.5-P21), NOD mice (2-wk-8-mo), pregnant mice (0–15 days post coitum), *ob/ob* mice (20-wk; n = 3, body weight  $68.6 \pm 2.4$  g, blood glucose level  $54.3 \pm 24.9$  mg/dL), *db/db* mice (20-wk; n = 3, body weight  $39.9 \pm 0.04$  g, blood glucose level  $421.7 \pm 21.8$  mg/dL) and wild-type mice (6-mo) (Fig. 2A). An increased ratio in  $\alpha$ -cells compared to  $\beta$ -cells was observed in E17.5 embryos to P12, in young prediabetic NOD mice (2–6 wk), and in pregnant mice at 9 and 13 dpc. A mild increase was also seen in *db/db* mice, but not in *ob/ob* mice.  $\alpha$ -cells in neonatal, pregnant, NOD, *ob/ob*, and *db/db* mice expressed PC1/3 in high frequency (Fig. 2B).

Regardless of the relative increase in the  $\beta$ - to  $\alpha$ -cell percentage as age increase, especially in neonates and NOD mice, the frequency of PC1/3 in both  $\beta$ - and  $\alpha$ -cells within each model remains fairly consistent. In pregnant mice, however, there is a slight decrease in both the frequency of  $\alpha$ -cells in islets and their overlap with PC1/3 as the mice age. An increase in  $\alpha$ -cell frequency among the various mouse models also did not necessarily correlate with an increase in the presence of PC1/3. Despite the higher frequency of  $\alpha$ -cells in neonates compared to in NOD mice, the overall overlap of PC1/3 and  $\alpha$ -cells detected was nearly the same in both models.

**Activation of PC1/3 in  $\alpha$ -cells resulting in the production of bioactive GLP-1.**  $\alpha$ -cells expressing PC1/3 in neonates, prediabetic NOD and pregnant mice were positive for GLP-1 (Fig. 3A). Note that antibodies used to detect glucagon and GLP-1 were specific for the biologically active forms, i.e., PG33-61 and GLP-1(7-36)amide, respectively. Co-localization of bioactive glucagon and GLP-1 in the same granules in  $\alpha$ -cells was further confirmed by double-immuno-gold particle stainings (Fig. 3B and Table 1). We further confirmed that both PC1/3 and GLP-1 were expressed in  $\alpha$ -cells (Fig. 4A). Schematic view of putative role of PC1/3 activation in  $\alpha$ -cells is shown in Figure 4B.

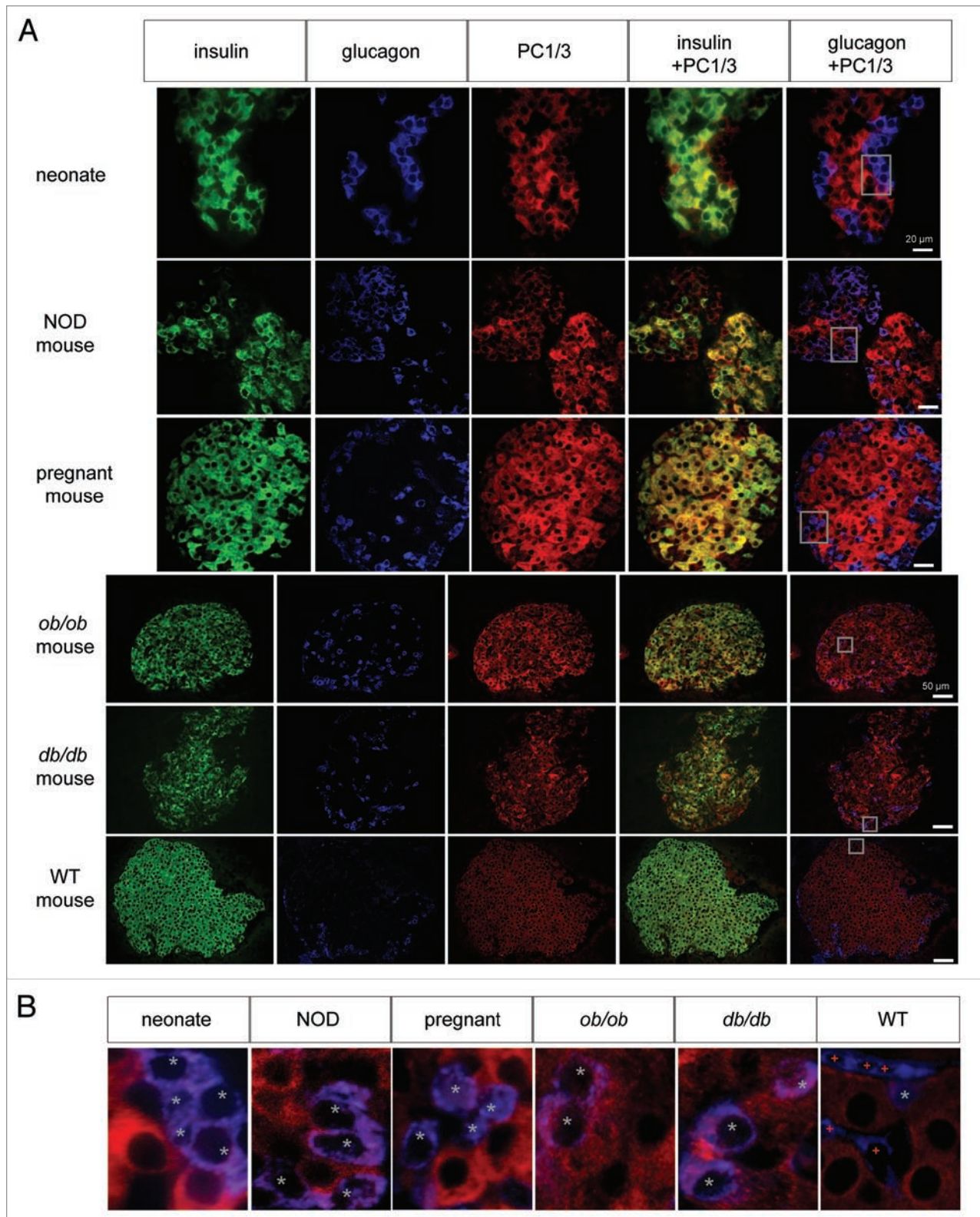
## Discussion

Post-translational regulation of endocrine hormone precursor processing is a complex process that is governed by tissue specific expressions of the prohormone convertase family, which includes PC1/3, PC2, PC4, PACE4, PC5/6 and PC7 (reviewed in ref. 7). Differential expression of each convertase with various affinities for multiple cleavage sites within a precursor enables a specific cell type to produce selected peptides, leaving the rest of a precursor unprocessed. The proglucagon precursor contains several bioactive polypeptides including glucagon, GLP-1 and GLP-2.<sup>8,9</sup> It is processed to glucagon by PC2 in pancreatic  $\alpha$ -cells, leaving GLP-1 and GLP-2 unprocessed within the major proglucagon fragment (MPGF) under normal conditions in adults.<sup>6,10</sup> In contrast, in intestinal L-cells, MPGF is processed to GLP-1 and GLP-2 by PC1/3, leaving glucagon unprocessed.<sup>10</sup>

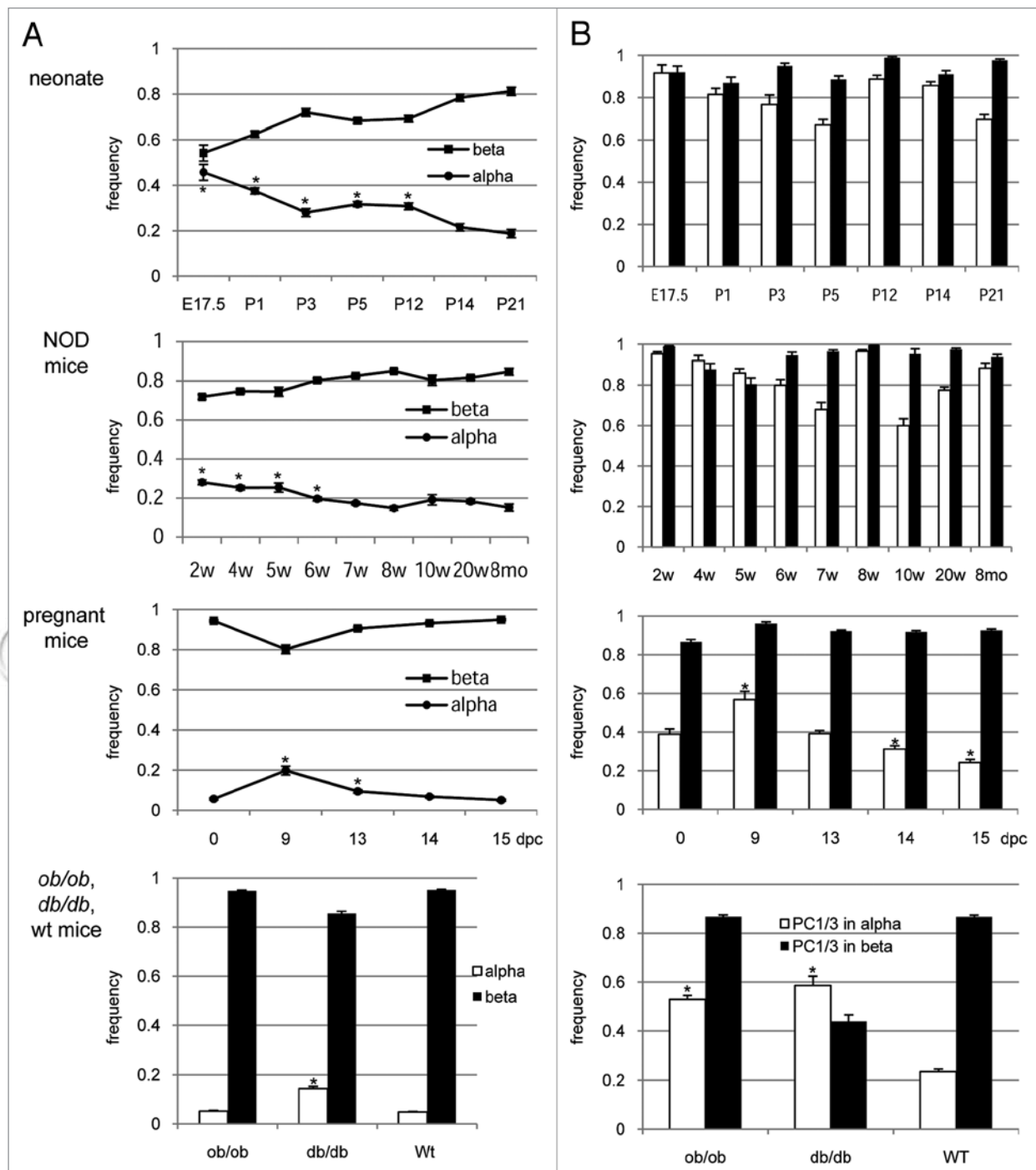
We have recently proposed a new model of islet formation by fission of endocrine cord-like structures formed in the embryonic and newborn pancreas, which occurs post-natally.<sup>3</sup>  $\alpha$ -cells appear to form the boundary of each islet-like mass within an elongated structure. These neonatal  $\alpha$ -cells express PC1/3, which results in the local production of a bioactive form of GLP-1 as we have shown in the present study. Activation of PC1/3 expression in  $\alpha$ -cells has been reported in the embryo<sup>11,12</sup> and in streptozotocin-induced diabetic mice.<sup>13</sup> We have also observed the expression of PC1/3 and GLP-1 in four mouse model of insulin resistance: (1) prediabetic non-obese diabetic (NOD) mice; (2) pregnant mice; (3) *ob/ob* mice; and (4) *db/db* mice. Taken together, differential temporal expression of PC1/3 in  $\alpha$ -cells can lead to the production of GLP-1 in specific physiological and pathological conditions.

The major source of ghrelin production is changed from embryos to adults in mice.<sup>14-16</sup>  $\alpha$ -cells in the embryonic pancreas synthesize both glucagon and ghrelin. However, as epsilon-cells emerge and are specialized in ghrelin-secretion, ghrelin expression in  $\alpha$ -cells is lost, and furthermore, the stomach becomes a major source of ghrelin in adults. The fact that PC1/3 processes the ghrelin precursor to ghrelin and obestatin<sup>17</sup> may suggest its involvement in the temporospatial expression of ghrelin. It further implies a potentially important role for ghrelin in  $\beta$ -cell proliferation, which could also be processed in  $\alpha$ -cells in specific conditions.

It is of great interest to determine what activates PC1/3 expression in specific cell types when needed. Our observations suggest that growth hormones in neonatal development, prolactin and placental lactogen during pregnancy (reviewed in ref. 18), fatty acids in obesity<sup>19</sup> and cytokines in prediabetic states<sup>20,21</sup> may be reasonable candidates. The conditional deletion of PC1/3 in mice should be a useful tool to elucidate mechanisms. Unfortunately, studies on global PC1/3-null mice show that it is a complex model, reflecting the enzyme's broad expression in multiple tissues and the involvement of many endocrine precursors.<sup>22</sup> The disruption of PC1/3 in mice caused dwarfism due to a defect in growth hormone releasing hormone processing, which was accompanied with multiple neuroendocrine peptide processing defects.<sup>22</sup> Interestingly, two cases of PC1/3 deficiency reported in human subjects exhibited severe obesity with other endocrine defects, including high circulating levels of proinsulin.<sup>23,24</sup> The obesity phenotype was mimicked by mice bearing a mutation in the PC1/3 gene.<sup>25</sup> It has been suggested that some remaining activity by point mutations (as opposed to a gene knockout) and differing efficiencies on different prohormones may lead to complicated phenotype presentations.<sup>25</sup> Similarly, global knockout of the GLP-1 receptor in mice, which our study suggests is the target of PC1/3 activation in  $\alpha$ -cells, may mask specific effects in pancreatic islets, since many studies in the field suggest the importance of the endocrine-cell network including possible compensations as discussed below. Therefore, the  $\beta$ -cell specific conditional deletion of the GLP-1 receptor, in combination with the  $\alpha$ -cell specific conditional disruption of PC1/3, should facilitate in clarifying this complexity.



**Figure 1.** Activation of PC1/3 expression in  $\alpha$ -cells. (A) Immunohistochemical analysis of PC1/3 expression in  $\beta$ - and  $\alpha$ -cells. Representative stainings for insulin (green), glucagon (blue), and PC1/3 (red) are shown in the following order: neonates (P6), NOD mouse (4 wk), pregnant mouse (13 days post coitum, or dpc), *ob/ob* mouse (20 wk), *db/db* mouse (20 wk) and wild-type mouse (6-mo). Note that glucagon-expressing  $\alpha$ -cells also express PC1/3 as in insulin-expressing  $\beta$ -cells except most of those in wild-type mice. (B) Enlarged regions from images in glucagon + PC1/3 column (rectangles) in (A) showing co-expression in  $\alpha$ -cells (asterisks). Note that most of the  $\alpha$ -cells in adult wild-type mice lack PC1/3 expression (marked with a symbol: +), though some  $\alpha$ -cells are positive (~20% at 6 mo examined; Fig. 2B).



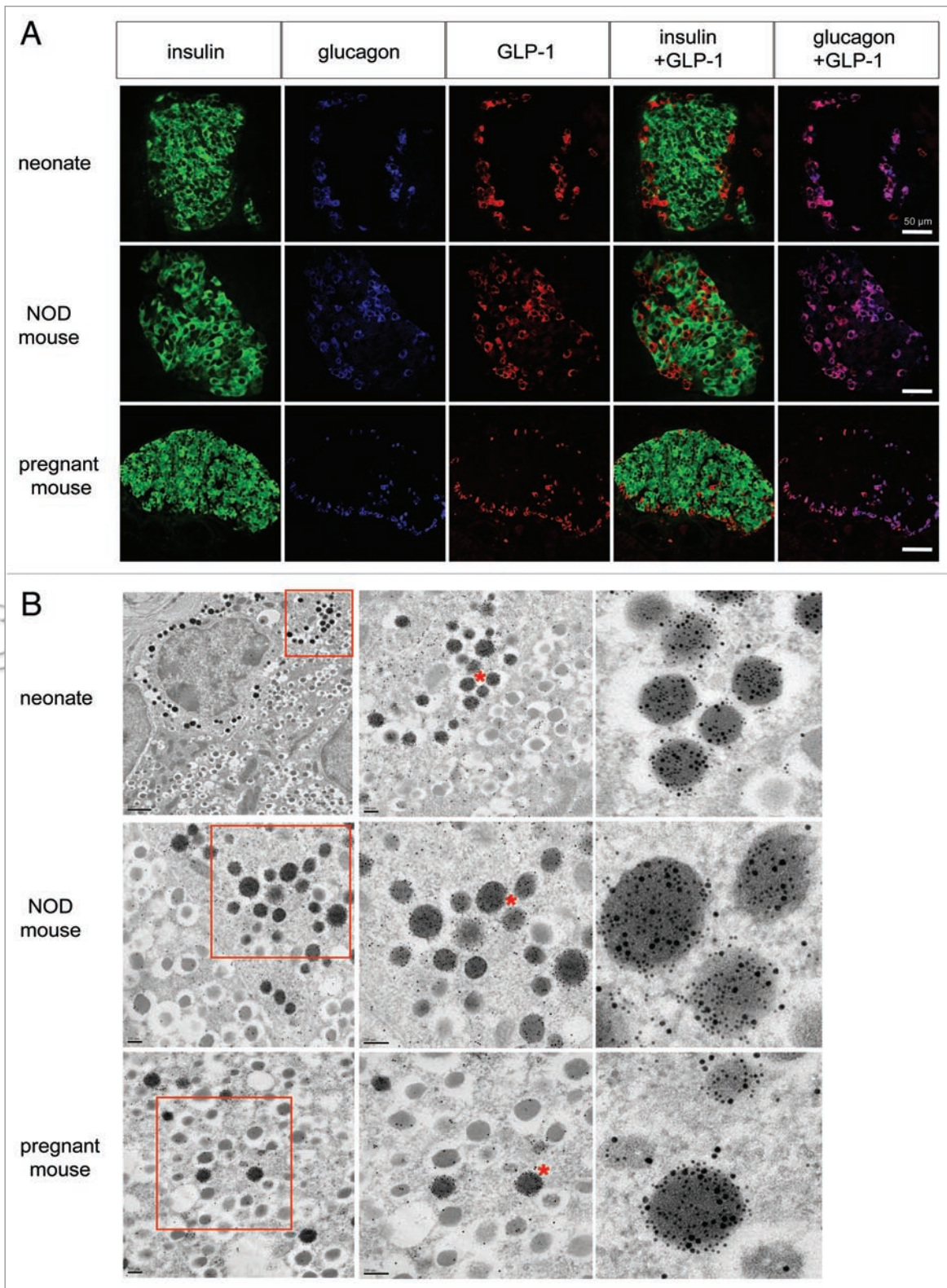
**Figure 2.** Time-course of changes in the  $\alpha$ -cell to  $\beta$ -cell ratio and PC1/3 expression. (A) Changes in the  $\alpha$ -cells to  $\beta$ -cells ratio in the developing pancreas (E17.5-P21), NOD mice (2-wk-8-mo), pregnant mice (0–15 dpc), ob/ob mice (20-wk), db/db mice (20-wk) and wild-type mice (6-mo). Asterisks (\*) denote  $p < 0.05$ . (B) Frequency of PC1/3 expression in  $\alpha$ - and  $\beta$ -cells.

In summary, the present study suggests a role for the  $\alpha$ -cell and  $\alpha$ -cell hyperplasia in the regulation of  $\beta$ -cell proliferation, and that the  $\alpha$ -cell may be the direct target of signals regulating  $\beta$ -cell proliferation. These signals lead to changes in  $\alpha$ -cell gene expression that trigger the biosynthesis and secretion of GLP-1, which implies the intrinsic intraislet mechanism of GLP-1 in the endocrine cell network. Further studies on the complex paracrine

and autocrine interactions among endocrine cells should lead to a better understanding of  $\beta$ -cell mass regulation.

### Materials and Methods

**Mice.** The following strains of mice were used: CD-1, non-obese diabetic (NOD) mice, ob/ob mice and db/db mice. All



**Figure 3.** GLP-1 expression in  $\alpha$ -cells. (A) Immunohistochemical analysis of GLP-1 expression in  $\alpha$ -cells. Representative stainings for insulin (green), glucagon (blue), and GLP-1(7-36)amide (red) are shown in the following order: neonate (P6), NOD mouse (4-wk), and pregnant mouse (14 dpc). (B) Immunoelectron microscopy. Representative double-immuno-gold particle stainings for glucagon (10 nm) and GLP-1(7-36)amide (15 nm) are shown in the following order: neonate (P16), NOD mouse (4-wk) and pregnant mouse (13 dpc). Areas in rectangles in the left panels are shown enlarged in the center. Images on the right are further magnified to identify particles of different sizes (red asterisks). Note that glucagon and GLP-1 are co-expressed in the same glucagon granules. Adjacent  $\beta$ -cells show characteristic insulin granules with less-dense cores and halos, which lack glucagon or GLP-1 labeling.

**Table 1.** Co-localization of glucagon and GLP-1 in glucagon granules

Condition	Specimen	Co-localization (%)	Number of granules
Development	Neonate (P3)	97.7 ± 0.8	328
Development	Neonate (P16)	96.7 ± 1.2	132
Inflammation/ Diabetes	Prediabetic (4 wk)	95.8 ± 0.9	427
Pregnancy	13 dpc	98.0 ± 1.1	140
Pregnancy	17 dpc	98.6 ± 0.9	187
Pregnancy	Post delivery	99.0 ± 0.7	236

procedures involving mice were approved by the University of Chicago Institutional Animal Care and Use Committee.

**Immunohistochemistry.** Mouse pancreata were excised, paraffin-embedded and cut 6 μm in thickness. Sections were stained with a polyclonal guinea pig anti-porcine insulin primary antibody (DAKO, Carpinteria, CA), a mouse monoclonal anti-human primary glucagon antibody (PG33-61; Sigma-Aldrich, St. Louis, MO), a polyclonal rabbit anti-glucagon-like peptide-1(7-36)amide (Abcam, Cambridge, MA) and a polyclonal rabbit anti-prohormone convertase 1/3. The primary antibodies were detected using a combination of Cy2, Cy5 and Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch Lab., West Grove, PA). Microscopic images were taken with an Olympus IX8 DSU spinning disk confocal microscope (Melville, NY). Fluorescent images were analyzed using ImageJ (rsbweb.nih.gov/ij/).

**Quantification.** Fluorescent images were analyzed using a macro routine written for ImageJ (rsbweb.nih.gov/ij/). Black and white masks covering the stained regions of each fluorescence channel were obtained using a partially automatic threshold determination. Areas where PC1/3 was co-expressed with glucagon and with insulin were determined using logical AND operations in ImageJ. To calculate the frequencies of PC1/3 co-expression, the areas where PC1/3 was co-expressed with glucagon or insulin was measured and then divided by the total area of glucagon or insulin expression, respectively.

**Electron microscopy.** For transmission electron microscopic analysis, a piece of pancreatic tissue was fixed with 4% PFA and 0.1% glutaraldehyde for 1 hr and embedded in resin using LR White (EM Sciences, Port Washington, PA). Sections were cut 80 nm in thickness. Glucagon and GLP-1 antibodies were detected with goat-anti mouse 10 nm and goat-anti rabbit 15 nm gold particles, respectively (EM Sciences).

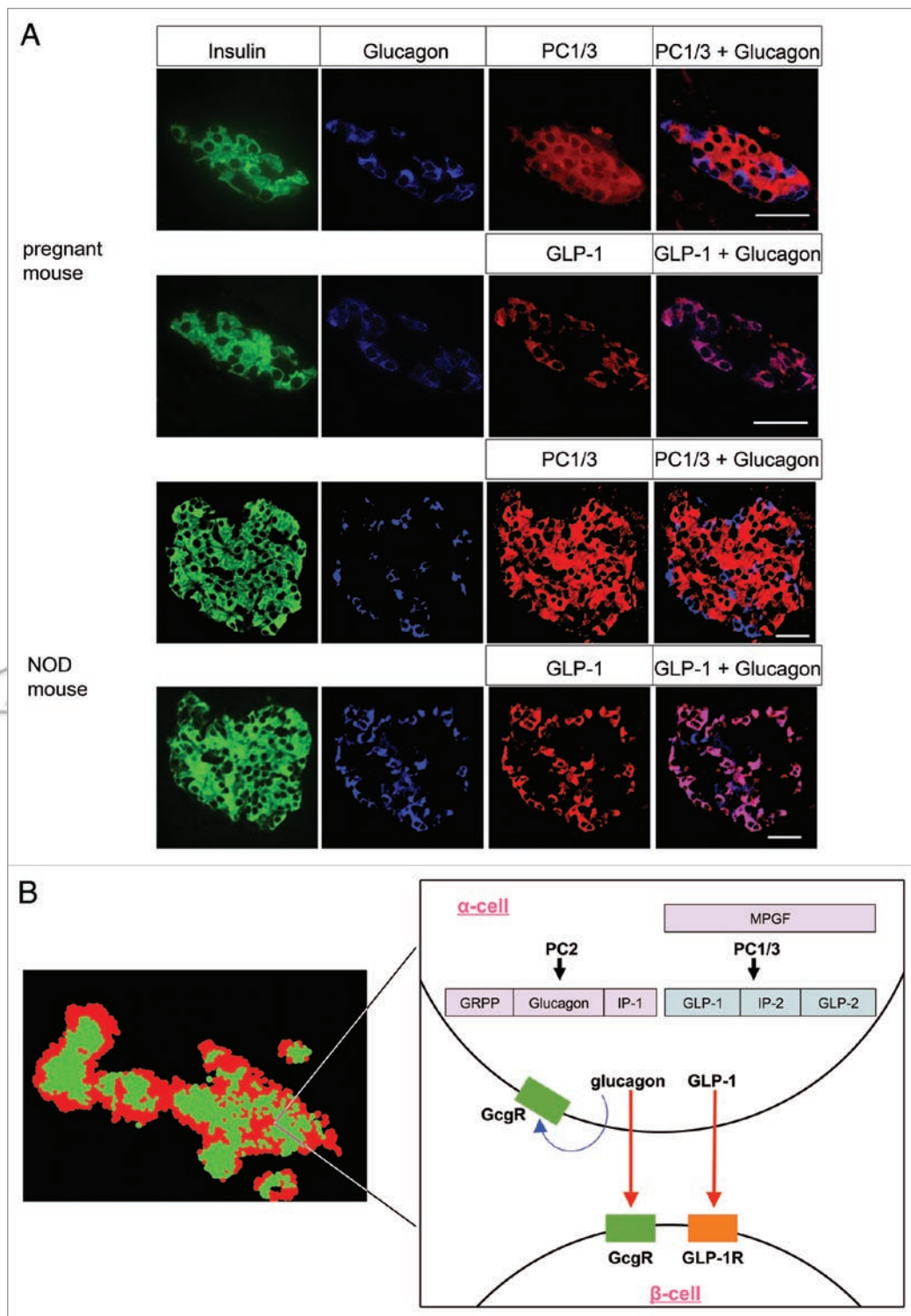
**Statistical analysis.** Data are expressed as mean ± SEM. Statistical analyses were performed using Student's t test. Differences were considered to be significant at  $p < 0.05$ .

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**Figure 4.** Co-localization of PC1/3 and GLP-1 in  $\alpha$ -cells. (A) Immunohistochemical analysis of PC1/3 and GLP-1. Representative stainings for insulin (green), glucagon (blue), PC1/3 (red in the upper) and GLP-1(7-36)amide (red in the lower) are shown in pregnant mouse (15 dpc) and NOD mouse (5-wk). Note that since both PC1/3 and GLP-1 primary antibodies are anti-rabbit, adjacent sections were used for each staining. Scale bars are 50  $\mu$ m. (B) Role of  $\alpha$ -cells in  $\beta$ -cell proliferation. Schematic view of putative interaction between  $\alpha$ - and  $\beta$ -cells is shown. Islet formation may occur in the neonatal pancreas by a process of fission in large stretches of interconnected islet structures at putative cleavage sites where  $\alpha$ -cells span these elongated structure.<sup>3</sup> These  $\alpha$ -cells express prohormone convertase (PC) 1/3. The absence of PC1/3 in adult  $\alpha$ -cells under normal conditions results in the production of glucagon leaving bioactive peptides unprocessed. GRPP: Glicentin-related pancreatic polypeptide; IP-1: intervening peptide-1; major proglucagon fragment (MPF); GLP-1: glucagon-like peptide-1; IP-2: intervening peptide-2; GLP-2: glucagon-like peptide-2. Expression of PC1/3 during development results in processing of proglucagon to GLP-1, which binds to the GLP-1R on  $\beta$ -cells stimulates  $\beta$ -cell proliferation.