Combination treatment of *db/db* mice with exendin-4 and gastrin preserves β-cell mass by stimulating β-cell growth and differentiation

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ABSTRACT

Aim/Introduction: Preservation of β-cell mass is crucial for maintaining long-term glucose homeostasis. Therapies based on incretin and its mimetics are expected to achieve this goal through various biological functions, particularly the restoration of β-cell mass. Here we tested the effects of gastrin and exendin-4 in type 2 diabetic animals.

Materials and Methods: The effects of exendin-4 and gastrin on β-cell function and mass were examined in 8-week-old *db/db* mice. INS-1 beta cells and AR42J cells were used to determine the molecular mechanism underlying the effects of the two agents. Immunohistochemistry, western blotting and RT-PCR assays were used to assess the biological effects of the two agents.

Results: Two weeks of combination administration of exendin-4 plus gastrin resulted in a significant improvement of glucose tolerance associated with a marked preservation of β-cell mass in *db/db* mice. Immunohistochemical analysis showed that such treatment resulted in the appearance of numerous irregularly-shaped small islets and single insulin-positive cells. While gastrin had little biological effect on INS-1 β-cells consistent with low expression of its intrinsic receptor on these cells, it caused differentiation of AR42J cells into insulin-producing cells. Co-stimulation with exendin-4 significantly enhanced gastrin-induced endocrine differentiation of AR42J precursor cells. These findings were further supported by enhanced expression of key genes involved in β-cell differentiation and maturation, such as neurogenin3 (Ngn3) and MafA.

Conclusions: These results suggest that combination treatment of *db/db* mice with exendin-4 and gastrin preserves β-cell mass by stimulating β-cell growth and differentiation. (J Diabetes Invest, doi: 10.1111/j.2040-1124.00044.x, 2010)

KEY WORDS: Gastrin, GLP-1, Type 2 diabetes

INTRODUCTION

Type 2 diabetes mellitus is a heterogeneous and polygenic metabolic disease[1]. The pathogenic mechanisms of this disease are attributed to two key factors: dysfunction of pancreatic β-cells and insulin resistance in target tissues[2,3]. Recent studies have suggested that age-dependent deterioration of type 2 diabetes is associated with progressive loss of β-cell function, which could be at least partly explained by loss of functional β-cell mass[4]. Over the past few decades, various therapeutic options have been proposed to maintain β-cell function and mass[5,6].

Among those therapeutic options, glucagon-like peptide 1 (GLP-1) and its mimetic compound, exendin-4, a new class of diabetic agents, exert their functions through multiple pathways, acting on the brain to induce satiety, inhibiting gastric emptying and glucagon secretion, and potentiating glucose-stimulated insulin secretion, all together leading to improved glucose homeostasis[7–12]. In addition, GLP-1 also has proliferative and anti-apoptotic effects on rodent β-cells[13–15]. Not only regulating replication and apoptosis of pre-existing β-cells, GLP-1 and exendin-4 also induce neogenesis of β-cells both *in vivo* and *in vitro*[14–17]. Thus, GLP-1-based therapy has attracted great attention as a therapeutic strategy for maintaining long-term glucose homeostasis by preserving β-cell mass.

In contrast, to identify signals that control growth and differentiation of pancreatic β-cells, many soluble factors with growth-promoting activity on β-cells have been extensively characterized. Growth factors that can stimulate β-cell replication include epidermal growth factor (EGF) and other EGF family members, such as heparin-binding EGF (HB-EGF), transforming growth factor-alpha (TGF-α) and betacellulin (BTC)[18–22]. Others, such as activin-A and hepatocyte growth factor (HGF), have been proposed to induce neogenesis of β-cells[23,24]. To date, several reports have been published to show that combination therapy with EGF plus gastrin stimulates the replication of rodent and human pancreatic β-cells, β-cell neogenesis and improves glucose tolerance in diabetic animal models[25–28]. Furthermore, recent studies reported that combination therapy with GLP-1 and gastrin induces β-cell neogenesis from pancreatic duct cells in human islets transplanted in immunodeficient

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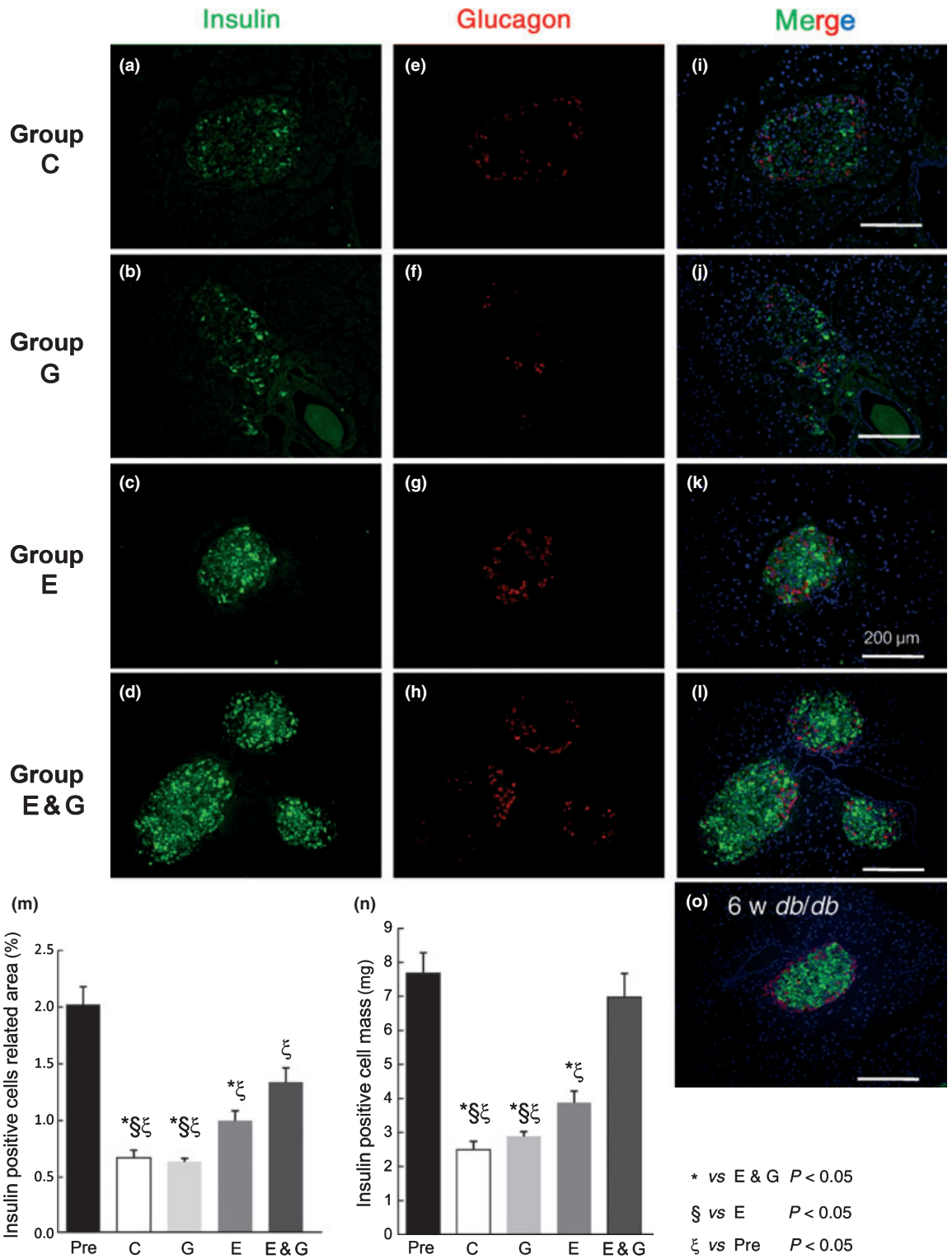


Figure 1 | Combination of exendin-4 and gastrin therapy preserves β-cell mass in *db/db* mice. Double staining for insulin (green, a–d) and glucagon (red, e–h) in pancreatic islets of 8-week-old *db/db* mice. Nuclei were labeled with DAPI (blue). (i–l) Merged images are shown. Six-week-old *db/db* mice were treated with (a,e,i) vehicle control (group C), (b,f,j) gastrin (group G), (c,g,k) exendin-4 (group E) and (d,h,l) exendin-4 plus gastrin (group E&G) for 2 weeks. (m) Proportion of insulin-positive cells in the pancreas (β-cell positive area/exocrine area). (n) Insulin-positive cell mass (proportion of insulin-positive cells in the pancreas × whole pancreas weight). (o) Six-week-old pretreatment *db/db* pancreas was stained for insulin and glucagon (group Pre). Data are mean ± SEM ($n = 6$). *$P < 0.05$ *vs* E&G group, §$P < 0.05$ *vs* E group. ξ$P < 0.05$ *vs* Pre group. Bar, 200 μm.

Figure 2 | Histological changes in the pancreas of *db/db* mice treated with exendin-4 plus gastrin. Pancreatic sections were stained for insulin (green), glucagon (red) and DAPI (blue). (a) Small and non-encapsulated islets and (b) single insulin-positive cells were markedly increased in exendin-4 plus gastrin (E&G)-treated mice. BrdU staining was carried out to assess cell proliferation in these islets. (c,d) Small islets in E&G-treated mice contained more proliferating cells (brown). (e,f) To assess whether BrdU-positive cells were insulin-positive cells, insulin (green) and BrdU (red) were co-labeled. (g) Proportion of BrdU-positive cells in islets. Data are mean ± SEM ($n = 6$). (h) Proportion of BrdU-positive cells in small islets composed of less than 100 cells ($n = 6$). (i) Mean cell number in islets ($n = 6$). (j) Distribution of islet size in each group. *$P < 0.05$ *vs* E&G group, §$P < 0.05$ *vs* E group. Co-administration of exendin-4 and gastrin increased the number of small islets. (k–p) Azan staining showed that the induced islets were non-encapsulated and irregular in shape. (k,l) Normal islets. (m–p) Non-encapsulated and irregular islet.

Table 1 | Effects of 2-week treatment on weight of whole pancreas

Treatment	Pancreas weight (mg)	*P*-value
Control	371.5 ± 5.3	
Exendin-4	388.5 ± 7.7	
Gastrin	454.0 ± 14.8	
Exendin-4 and gastrin	553.5 ± 23.7	$P < 0.05$ *vs* exendin-4 $P < 0.05$ *vs* control

Data are mean ± SEM of five mice in each group.

diabetic mice, although the underlying mechanisms remain largely unknown[29].

Given that the growth-promoting action of GLP-1 on β-cells is partly mediated by signaling pathways downstream of epidermal growth factor receptor (EGFR)[30], we hypothesized that combined administration of gastrin with exendin-4 could have beneficial therapeutic effects on type 2 diabetic mice through the stimulation of β-cell replication and/or differentiation. The results of the present study showed that the combined administration of gastrin with exendin-4 in diabetic *db/db* mice resulted in the preservation of β-cell mass through increased β-cell proliferation and the stimulation of β-cell differentiation from non-insulin producing precursors by activating neurogenin3 (Ngn3), an essential factor for endocrine differentiation.

MATERIALS AND METHODS

Animals

Five-week-old female *db/db* mice (BKS.Cg-m+/+Lepr^{db}/Jcl) were purchased from Clea Japan (Tokyo, Japan). The study protocol was reviewed and approved by the Animal Care and Use Committee of Juntendo University. Mice were housed under controlled light (14 h light/10 h dark) and temperature conditions, and had free access to standard rodent chow and water.

Study Design of Animal Experiments

All animal experiments were initiated in mice at 6 weeks-of-age after glucose tolerance was assessed by an intraperitoneal glucose tolerance test (IPGTT) with glucose at 0.5 g/kg of bodyweight, as described previously[31]. Based on the results of IPGTT, the mice were divided into four groups (E, exendin-4; G, gastrin; E&G, exendin-4 plus gastrin; and C, control) in a manner that the average tolerance of one group was not significantly different from that of the others. Phosphate-buffered saline (PBS) or exendin-4 (100 μg/kg i.p.; Sigma-Aldrich, Tokyo, Japan) and/or human gastrin-I (1 mg/kg; Sigma-Aldrich, Tokyo, Japan) were injected once daily at 09.00 for 14 days.

Immunohistochemical Analysis of Pancreatic Sections

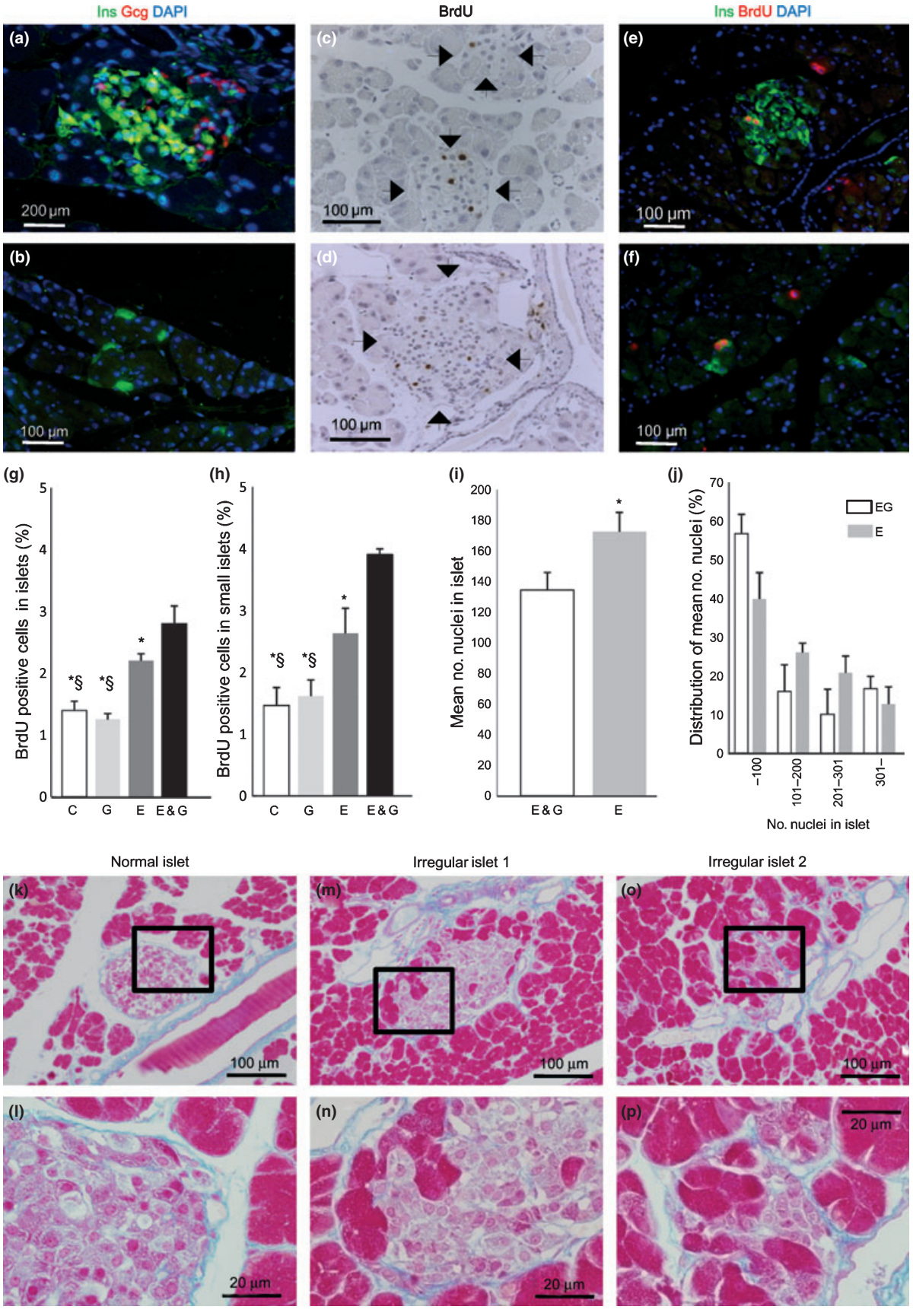
After anesthetization of 8-week-old mice, the pancreas was removed after cardiac perfusion and fixed overnight in a solution of 4% paraformaldehyde at 4°C. The fixed tissue was embedded in paraffin and then cut into 4-μm-thick sections and mounted on slides. Immunohistochemical analysis was carried out using each primary antibody (listed in Table S1). The first antibody was applied to the specimen and incubated at 4°C overnight. Then, the second antibody was applied at room temperature for 60 min. All secondary antibodies were purchased from commercial sources (listed in Table S1). The area of insulin-positive cells (%) relative to whole pancreas area was estimated from six mice per group, using six immunostained sections per mouse, with each section separated by at least 200 μm. The method used for calculation of insulin-positive cells has been described in detail previously[31].

Quantitative RT-PCR Analysis for Genes Expressed in AR42J Cells

Total RNA was extracted from AR42J cells cultured for 3 days with exendin-4 (1 nmol/L) and/or rat gastrin-I (10 nmol/L). The mRNA were extracted using RNeasy mini kit (Qiagen, Tokyo, Japan). Then, cDNA was synthesized by using a High Capacity cDNA Reverse Transcription kit with adjunctive random primer (ABI, Tokyo, Japan). To investigate the expression level of each mRNA, cDNA were amplified using power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) or Quantitect Probe PCR kit (Qiagen). Quantitative RT-PCR was carried out on ABI 7500 real-time PCR system (Applied Biosystems). The relative abundance of mRNA was calculated by the comparative cycle of threshold (CT) method with β-actin mRNA as the invariant control. The primers used for PCR are listed in Table S2.

Western Blot Analysis

AR42J and INS-1 cells seeded in 6-well plates were incubated with exendin-4 (1 nmol/L) and/or rat gastrin-I (10 nmol/L) for 5–60 min for analysis of signaling pathways or 3 days for detection of insulin secretion. After washing three times with ice-cold PBS, cells were lysed[31]. Then 30 μg of total cell extracts prepared from these cells was separated on SDS-polyacrylamide gel, transferred to a polyvinylidene fluoride (PVDF) membrane



(Millipore, Tokyo, Japan), and incubated overnight at 4°C with specific antibodies (described in Table S1).

Statistical Analysis

Values were expressed as mean ± SEM. Differences between groups were analyzed by one-way analysis of variance (ANOVA) with correction for different variance whenever appropriate. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Combined Exendine-4 Plus Gastrin Therapy Preserved β-Cell Mass in *db/db* Mice

Drug treatment was initiated in *db/db* mice at 6-weeks-of-age before the onset of overt diabetes. After 2 weeks of drug administration, whole pancreas was harvested at 8-weeks-of-age and subjected to histological analysis (Figure 1a–l). As described previously[32], islets of non-treated *db/db* mice contained markedly few and scattered insulin-positive cells. In mice treated with exendin-4 plus gastrin (E&G) or exendin-4 (E), the number of insulin-positive cells and intensity of insulin staining in β-cells were relatively maintained.

The insulin-positive area in mice treated with E&G or exendin-4 alone was significantly greater than in mice treated with gastrin or untreated control mice. The insulin-positive area relative to whole exocrine pancreas (A [%]) in mice treated with E&G was significantly greater than mice treated with exendin-4 (Figure 1m). Whole pancreas weight (B [mg]) in mice treated with E&G was significantly greater than in mice treated with exendin-4 alone (Table 1). Consistent with these findings, BrdU-labeling showed that treatment with E&G stimulated mitosis of acinar and ductal cells in addition to cells in islets (data not shown). Accordingly, β-cell mass (calculated as B × A [mg]) in mice treated with E&G was markedly greater than mice treated with exendin-4 alone or gastrin alone and the β-cell mass was largely preserved as it was at the age of 8 weeks (Figure 1n).

Histological Features of the Pancreas of *db/db* Mice Treated with Exendin-4 Plus Gastrin

To gain insight into the observation that β-cell mass of mice treated with E&G was larger than that of exendin-4 treated mice, more detailed morphometric analyses were carried out. Numerous small islets, containing <100 cells on the sectional plane, were found in *db/db* mice treated with E&G (Figure 2). Of note, many of these small islets were noncapsulated, as determined by azan staining (Figure 2k–p). A number of insulin-positive cells were spindle-shaped with decreased cell–cell contact, as if they were prepared to migrate between acinar cell clusters (Figure 2a). In addition, single, extra-insular β-cells were frequently noted in E&G-treated mice (Figure 2b). These observations were consistent with the previous reports on the effect of gastrin on the pancreas[33,34], suggesting that the increase in small islets and small β-cell clusters contributed to the increased β-cell mass in E&G-treated *db/db* mice and that the appearance of these structures could be an indication of increased β-cell regeneration. To determine the cell-cycle status of islet cells, the S phase cells were pulse-labeled before fixation (20-h labeling period), followed by immunostaining using anti-BrdU antibody (Figure 2c–f, Appendix S1). Frequent co-labeling with BrdU and insulin indicated that at least some portion of the proliferating cells were designated insulin-producing cells, in response to growth factor stimulation (Figure 2e,f). Furthermore, the numbers of BrdU-positive cells were significantly higher in islets of *db/db* mice treated with E&G or exendin-4 alone than those treated with gastrin alone or untreated mice (Figure 2g). On careful examination of small islets (with <100 cells), the frequency of BrdU incorporation into islets was even more pronounced in E&G-treated *db/db* mice (Figure 2h). Furthermore, the mean number of cell per islet was significantly less in mice treated with E&G than in exendin-4 treated mice (Figure 2i). Islet size tended to be smaller in E&G-treated mice (Figure 2j).

Effect of Exendin-4 Plus Gastrin Therapy on Glucose Tolerance

The above results indicated that gastrin enhanced exendin-4-mediated an increase in islet mass. To assess the functional outcome of the above morphological and immunohistochemical changes, IPGTT was carried out to evaluate glucose tolerance. Because of its resistance to DPP-IV, a GLP-1 degradation enzyme, exendin-4 has a longer half-life period than native GLP-1[35]. To avoid the acute incretin effect of exendin-4[36], all experiments were carried out 24 h after the last injection to ensure clearance of the drug from the circulation. Glucose tolerance and insulin secretion in the four experimental groups were within comparable range at the initiation of treatment (Figure 3a,c). On day 14 of the treatment, vehicle-treated control mice showed significantly elevated fasting glucose levels and marked hyperglycemia after glucose challenge (Figure 3b), suggestive of the onset of overt diabetes. Consistent with the previous report[14], exendin-4 significantly reduced fasting glucose levels compared with vehicle-treated mice and suppressed glucose excursions, which was associated with increased insulin secretion (Figure 3d). Administration of gastrin alone showed a limited improvement in glucose tolerance. There was no additional blood-glucose-lowering effect for gastrin added on exendin-4 in this setting.

Little Evidence of Biological Effects Elicited by Gastrin on β-cells

The aforementioned results showed that E&G treatment further increased β-cell mass compared with exendin-4 alone, whereas gastrin alone had almost no effect on β-cell mass or glucose tolerance (Figures 1 and 3). Molecular mechanisms underlying the β-cell preserving effects of the addition of gastrin on exendin-4 might include two possibilities: (i) gastrin is capable of promoting exendin-4-induced β-cell proliferation; or (ii) gastrin can direct the differentiation of adult endocrine progenitors towards β-cell fate in collaboration with exendin-4. The former possibility is unlikely, because CCK2R is not expressed in β-cells[34]. Indeed, the addition of gastrin to INS-1 β-cell cultures caused

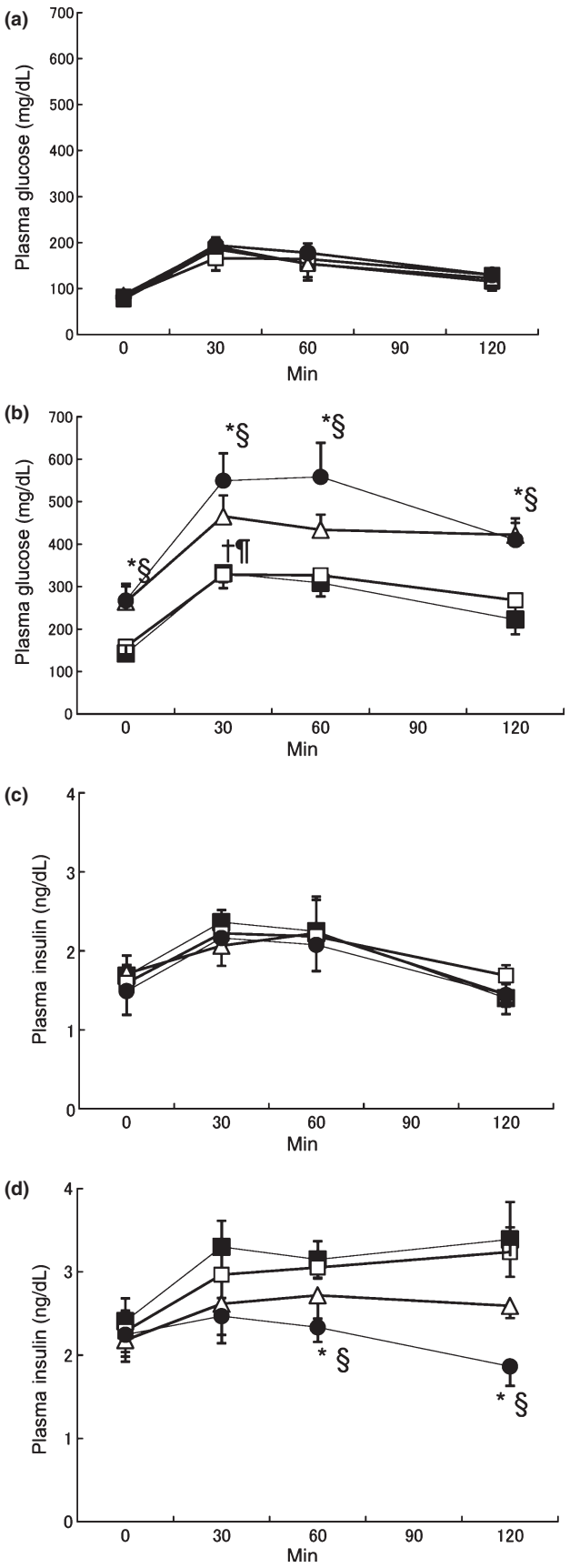


Figure 3 | Effects of exendin-4 plus gastrin combination therapy on glucose tolerance. Plasma glucose and insulin concentrations in *db/db* mice measured during IPGTT (0.5 g glucose/kg) before and after 2 weeks of treatment with exendin-4 (100 μg/kg = 24 nmol/L/kg) and/or human gastrin-I (1000 μg/kg = 477 nmol/L/kg), or vehicle (PBS) only. (a) Plasma glucose levels in 6-week-old mice before treatment. (b) Plasma glucose levels at 8 weeks after treatment. (c) Plasma insulin levels at 6 weeks before treatment. (d) Plasma insulin levels at 8 weeks after treatment. Solid circles, vehicle-treated control mice (C group); open squares, mice treated with exendin-4 (E group); open triangles, mice treated with gastrin (G group); solid squares, mice treated with exendin-4 plus gastrin (E&G group). Data are mean ± SEM (n = 6). *P < 0.05 C group *vs* E&G group, §P < 0.05 C group *vs* E group, †P < 0.05 G group *vs* E&G group, ¶P < 0.05 G group *vs* E group.

no phosphorylation of intracellular signaling molecules, including p42/44 MAPK, CREB, Akt and p38-MAPK, whereas exendin-4 efficiently phosphorylated these proteins as reported previously[37,38] (Figure S1). Furthermore, the combination of E&G was not different from the effect of exendin-4 alone on phosphorylation. Similarly, quantitative RT-PCR analysis showed that gastrin alone or the addition of gastrin on exendin-4 did not alter mRNA levels for a couple of GLP-1-responsive genes, such as IRS-2 and Cyclin D1 (Figure S1). Based on these findings, we hypothesized that gastrin induces β-cell differentiation, rather than the replication of pre-existing β-cells. To test this possibility, we next assessed whether gastrin in combination with exendin-4 could enhance β-cell differentiation programs, by using AR42J, a putative pancreatic progenitor cell line[16,23,39].

Insulin Expression in Pancreatic Precursor Cell Line AR42J Treated with Exendin-4 and/or Gastrin

Previous studies reported that treatment of AR42J cells, a tumor cell line showing pancreatic progenitor-like properties, with GLP-1/exendin-4 induced insulin-producing cells[16,17]. In preliminary experiments, the optimal concentrations of exendin-4 and gastrin for induction of efficient phosphorylation of p42/44 MAPK in AR42J were found to be 1 and 10 nmol/L, respectively (data not shown). To assess whether or not exendin-4 and/or gastrin can generate insulin-producing cells from AR42J progenitors, cells were treated with 1 nmol/L exendin-4 and/or 10 nmol/L gastrin for various intervals followed by immunostaining with anti-insulin antibody. Optimal induction of insulin-positive cells was observed when AR42J cells were incubated for 3 days with E&G (Figure 4a–d). The proportion of insulin-positive cells in the exendin-4 or gastrin-treated group was significantly higher than untreated control cells. Importantly, E&G co-stimulation resulted in a significant increase in the proportion of insulin-positive cells than monotherapy with exendin-4 or gastrin. Western blot analysis further confirmed that insulin protein was indeed synthesized in AR42J cells cultured with exendin-4 or gastrin and that insulin protein levels were synergistically increased by co-stimulation with the two agents (Figure 4h).

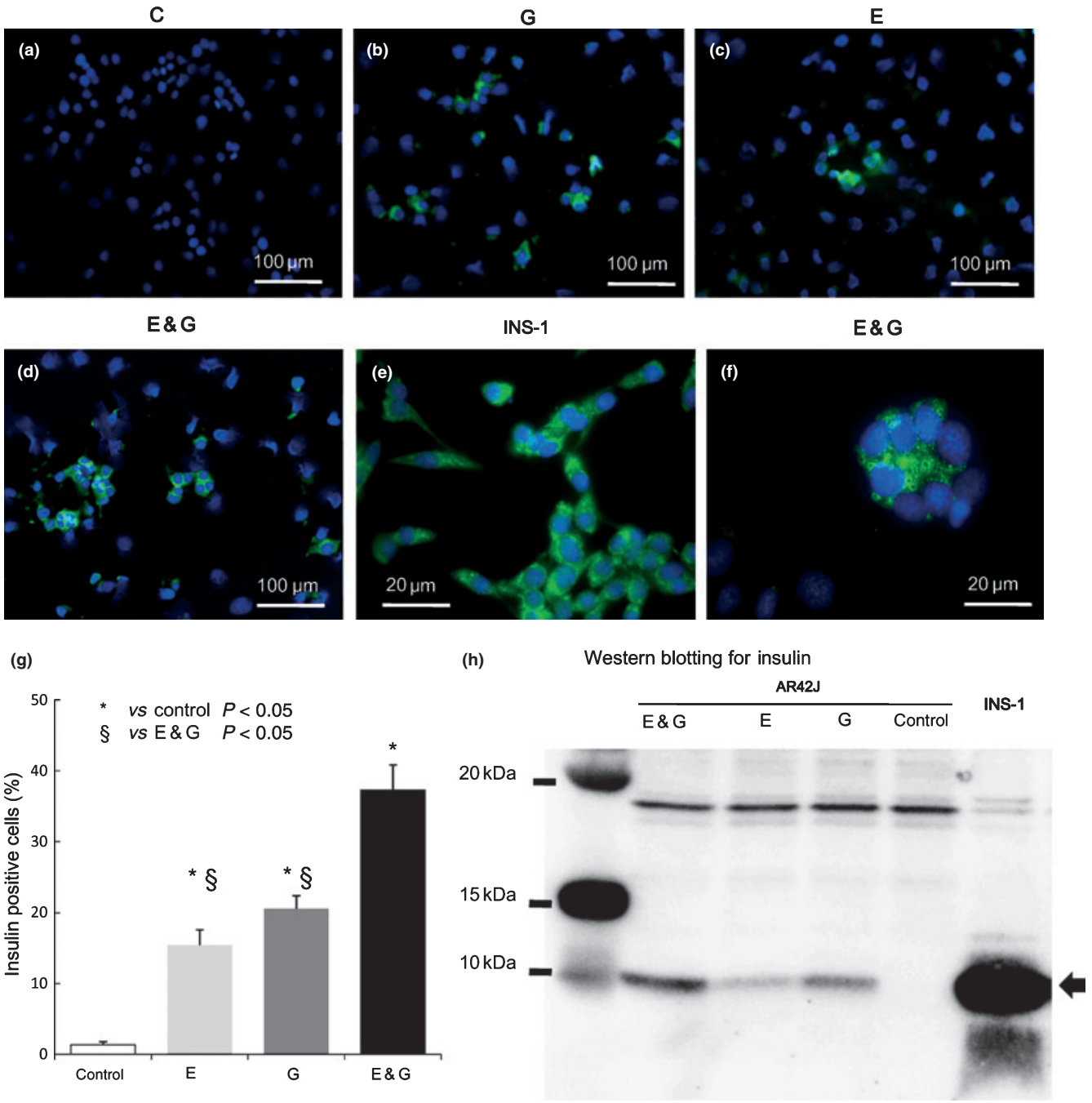


Figure 4 | (a–d,f) Insulin expression in pancreatic precursor cell line AR42J treated with exendin-4 and/or gastrin. AR42J cells treated for 72 h with exendin-4 (1 nmol/L) and/or gastrin (10 nmol/L) were stained with anti-insulin antibody (green) and DAPI (blue). (a) Untreated AR42J cells (C). (b) Gastrin-treated AR42J cells (G). (c) Exendin-4-treated AR42J cells (E). (d,f) AR42J cells treated with exendin-4 plus gastrin (E&G). (e) INS-1 insulinoma cells as a positive control. (g) Percentage of insulin-positive cells in each group. Data are mean ± SEM (n = 3). *P < 0.05 *vs* C group, §P < 0.05 *vs* E&G group. (h) Western blot analysis was carried out to assess insulin content of AR42J cells treated with exendin-4 and/or gastrin for 3 days. INS-1 insulinoma cells were used as a positive control. Representative data of three experiments with similar results.

Gene Expression in AR42J Cells Treated with Exendin-4 and/or Gastrin

To investigate the mechanisms underlying the generation of insulin-producing cells from AR42J cells, the expression of several genes involved in the β-cell differentiation pathway was assessed by quantitative RT-PCR. (Figure 5, n = 8–12 each). The expression levels of insulin I+II, Ngn3, NeuroD, and MafA were significantly upregulated by gastrin. Gastrin-induced

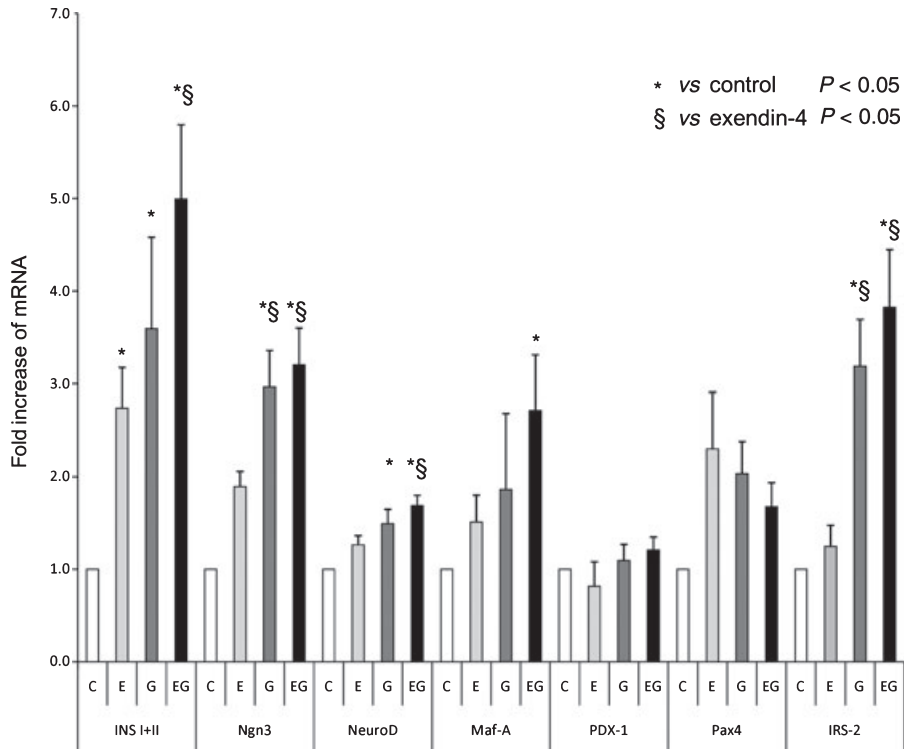


Figure 5 | The mRNA expression levels of various genes in AR42J cells incubated with exendin-4 and/or gastrin. AR42J cells were incubated for 3 days with exendin-4 (1 nmol/L) and/or gastrin (10 nmol/L). Subsequently, the mRNA expression levels of several genes related to β-cell differentiation were assessed by quantitative RT-PCR. C, non-treated AR42J control cells; E, exendin-4-treated AR42J cells; E&G, AR42J cells treated with exendin-4 plus gastrin; G, gastrin-treated AR42J cells. Data are mean ± SEM (n = 8–12 each). *P < 0.05 *vs* C group, §P < 0.05 *vs* E group.

upregulation of insulin I+II was further enhanced by concomitant stimulation with exendin-4. Although it is known that PDX-1 plays an important role in β-cell differentiation and that exendin-4 enhances its expression[17,40], PDX-1 was already abundantly expressed in untreated AR42J cells and growth factor stimulation did not augment its expression. Expression of IRS-2, which is important for β-cell growth[41,42], was also significantly activated by gastrin.

Gastrin Triggers P38 MAPK-Ngn3 Activation in AR42J Cells

Western blot analysis was carried out to determine whether exendin-4 and/or gastrin activate distinct signaling pathways in AR42J cells. P42/44 MAPK, CREB and p38 MAPK were phosphorylated by exendin-4 and/or gastrin treatment (Figure 6). The addition of gastrin to AR42J induced phosphorylation of p42/44 MAPK and CREB at levels greater than with exendin-4, but there were no synergistic or additive effects by exendin-4 and gastrin. Phosphorylation of Akt was also upregulated by E&G. While phosphorylation of p38 MAPK was not induced by administration of exendin-4 alone, its phosphorylation was observed in AR42J cells treated with E&G or gastrin alone (Figure 6). We have previously reported the involvement of p38 MAPK activation in the induction of Ngn3 and subsequent endocrine differentiation of AR42J cells in response to HGF plus

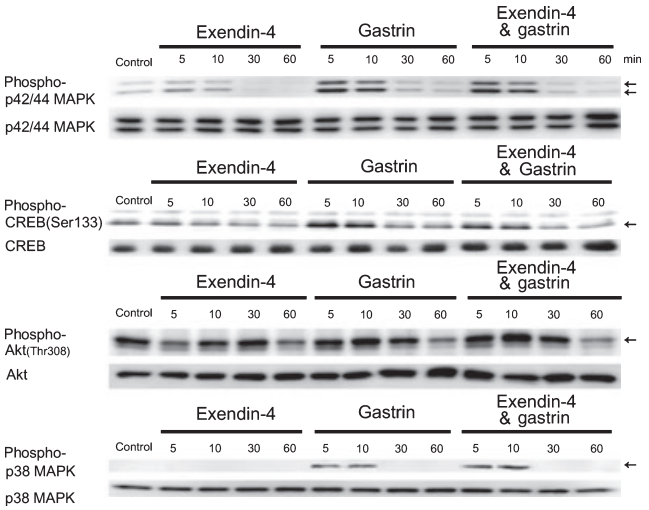


Figure 6 | Signals initiated by exendin-4 and gastrin in AR42J cells. Western blot analyses were carried out to determine the intracellular signals stimulated by exendin-4 (1 nmol/L) and/or gastrin (10 nmol/L) in AR42J cells. Representative immunoblots of phosphorylated p42/44 MAPK and total p42/44 MAPK, phosphorylated CREB at residue Ser133 ($CREB^{Ser133}$) and total CREB, phosphorylated Akt at residue threonine 308 (Akt^{Thr308}) and total Akt, and phosphorylated p38-MAPK and total p38-MAPK, at 5–60 min. Thirty microgram of total protein was applied in each lane. Representative data of three experiments each with similar results.

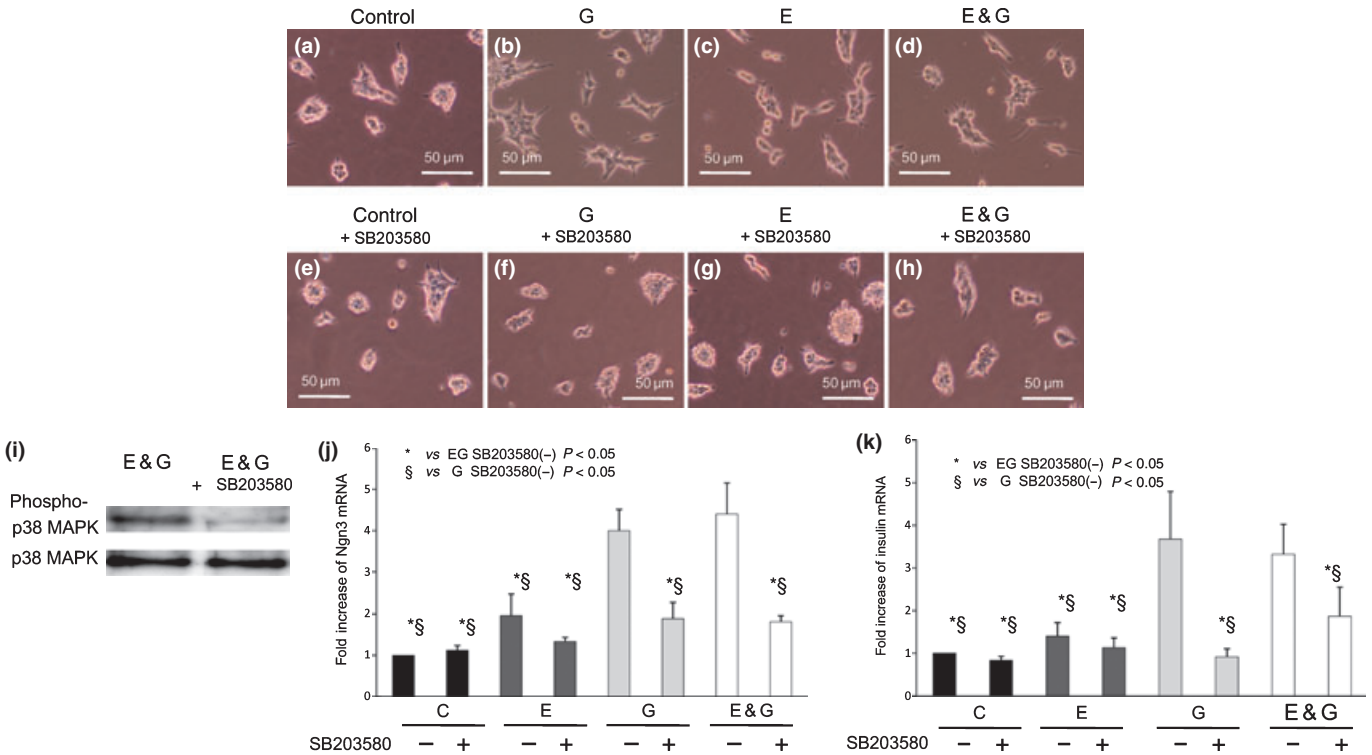


Figure 7 | SB203580, a p38-MAPK inhibitor, inhibited endocrine differentiation of AR42J cells induced by exendin-4 and/or gastrin. (a–h) Morphological effects of drug administration. (a) AR42J cells treated with vehicle only, (b) AR42J cells treated with gastrin, (c) AR42J cells treated with exendin-4, (d) AR42J cells treated with exendin-4 and gastrin, (e) AR42J cells treated with vehicle and SB203580, (f) AR42J cells treated with gastrin and SB203580, (g) AR42J cells treated with exendin-4 and SB203580, (h) AR42J cells treated with exendin-4, gastrin and SB203580. (i) AR42J cells were incubated with SB203580 for 1 h, and treated with exendin-4 and gastrin. Western blotting analysis was carried out to evaluate p38-MAPK activation. Phosphorylated and total p38-MAPK were shown. Representative data of three experiments with similar results was indicated. (j,k) AR42J cells were incubated with exendin-4 and/or gastrin for 72 h, with or without SB203580. Quantitative RT-PCR was carried out to determine the expression level of (j) Ngn3 mRNA and (k) insulin mRNA. Solid bars: vehicle, with or without SB203580, -treated AR42J cells; dark gray bars: exendin-4, with or without SB203580, -treated AR42J cells; light gray bars: gastrin, with or without SB203580, -treated AR42J cells; open bars: exendin-4 and gastrin, with or without SB203580, -treated AR42J cells. Data are mean ± SEM ($n = 5$). *$P < 0.05$ *vs* exendin-4 and gastrin, without SB203580, -treated AR42J cells, §$P < 0.05$ *vs* gastrin, without SB203580, -treated AR42J cells.

activin-A[24]. SB203580, a p38 MAPK inhibitor, abolished activation of Ngn3 and insulin in AR42J cells induced by gastrin or E&G. This inhibition of Ngn3 activation was coincident with the suppression of neurite extension, the earliest morphological change in AR42J cells undergoing endocrine differentiation[23] (Figure 7). These results suggest that β-cell differentiation induced by E&G is mediated by p38 MAPK-dependent Ngn3 activation.

Ngn3 Expression in *db/db* Mice Treated with Exendin-4 and Gastrin

As described above, *in vitro* analysis using AR42J cells showed that treatment of endocrine precursor cells with E&G could induce insulin-producing cells through Ngn3 expression. We investigated whether or not activation of Ngn3 is also observed in a *in vivo* setting, as shown previously in the ductal ligation models[33]. Quantitative RT-PCR analysis using mRNA isolated from whole pancreas showed that 1-week treatment with gastrin or E&G, but exendin-4 alone, induced Ngn3 expression (fivefold induction compared with vehicle-treated controls) in *db/db* mice (Figure 8, Appendix S1).

DISCUSSION

As reported previously[14], treatment of *db/db* mice with exendin-4 significantly preserved β-cell mass and ameliorated hyperglycemia. Whereas gastrin monotherapy showed no beneficial effects on glucose tolerance or pancreas histology[43], the combination therapy of exendin-4 plus gastrin induced a marked increase in β-cell mass, associated with numerous irregular and non-encapsulated islets and solitary β-cells (Figure 3). Such islets and β-cells, which appear to be 'regenerated' β-cells, were also observed in diabetic rats treated with exendin-4[15]. We found similar small islets and β-cell clusters in mice treated with exendin-4 alone, but these small clusters were more robustly induced in mice co-treated with gastrin. In such small islets, a substantial number of β-cells and β-cell precursors seemed to be actively

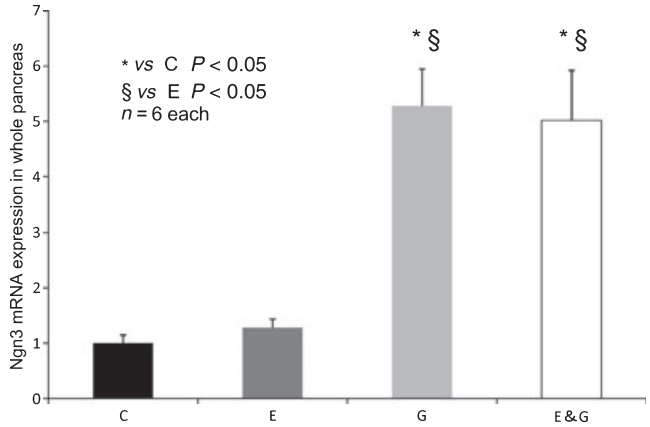


Figure 8 | Ngn3 expression in *db/db* mice treated with exendin-4 and gastrin. Six-week-old *db/db* mice were treated with exendin-4 (100 μg/kg/day) plus gastrin (1000 μg/kg/day) for 1 week. C, vehicle-treated *db/db* mice; E, *db/db* mice treated with exendin-4; E&G, *db/db* mice treated with exendin-4 plus gastrin; G, *db/db* mice treated with gastrin. Bars represent fold increase of Ngn3 mRNA in whole pancreas compared with vehicle-treated controls. Data are mean ± SEM (n = 12). *P < 0.05 *vs* C group, §P < 0.05 *vs* E group.

proliferating (Figure 2). Apoptotic islet cells were hardly observed in islets of *db/db* mice, as examined by TUNEL assay and cleaved caspase-3 staining at least within the time window (8–10 weeks-of-age; data not shown)[44]. Similar results were reported previously by Uchida *et al.*[45], whereas some other studies reported a substantial number of apoptotic cells in islets of *db/db* mice[14]. Based on the rare observation of apoptotic β-cells in *db/db* mice, we concluded that E&G treatment preserved β-cell mass by enhancing β-cell induction and proliferation rather than preventing β-cell apoptosis. Despite the significant preservation of β-cell mass by E&G, such treatment did not cause further improvement in glucose tolerance (Figure 3b). Irregularly shaped islets driven by E&G lack islet-capsules, which is usually associated with a capillary network surrounding an islet. As a result of the poorly developed capillary networks, the newly formed islets might be functionally immature at least at 8-weeks-old.

Consistent with previous reports that CCK2R, the endogenous receptor for gastrin, is not expressed in β-cells[34,46], gastrin failed to induce protein phosphorylation of key signal effectors or expression of genes associated with cell proliferation in INS-1 cells, even when combined with exendin-4 (Figure S1). These results are in agreement with the lack of effect of gastrin monotherapy on intra-islet β-cell proliferation[34,43] (Figure 1). These *in vitro* and *in vivo* findings collectively support the conclusion that the lack of the stimulatory effect of gastrin on mature β-cells (e.g., to induce biological responses, such as cell proliferation) is probably as a result of the lack of functional receptors on such cells. In contrast, gastrin efficiently stimulated AR42J cells to induce insulin-producing cells, and exendin-4 was able to enhance this effect (Figure 4). AR42J cells were originally isolated as a non-endocrine, pancreatic acinar-like cancer cell line. The combination of growth factors, such as activin-A plus betacellulin or HGF, is reported to stimulate AR42J cell to give rise to insulin-producing cells, thus AR42J possesses pancreatic progenitor-like properties[23,24]. GLP-1 and exendin-4 were reported to be among the factors that can induce differentiation of insulin-producing cells from mouse endocrine progenitors[15] or from AR42J cells[16,17]. Our *in vitro* analysis identified gastrin as a potent β-cell inducing factor capable of activating a battery of genes involved in the β-cell differentiation pathway (Figure 5). Given its failure in driving proliferation of matured β-cell, the *in vivo* effect of gastrin on preservation of β-cell mass when used with exendin-4 is likely to be mediated, at least in part, through its capability to induce a β-cell differentiation program.

Because both GLP-1R and CCK2R belong to G-protein coupled receptors (GPCRs), they are expected to operate through similar signaling pathways in AR42J cells. Activation of p38 MAPK was, however, noted only in gastrin-treated cells, a finding also supported by a previous study[47] (Figure 6). In this regard, we reported previously that activation of p38 MAPK is implicated in the induction of Ngn3 in activin-A plus HGF-treated AR42J cells[24]. Efficient activation of p38 MAPK in E&G-treated AR42J cells (Figure 6) might account for the induction of β-cell differentiation associated with Ngn3 activation. While it remains controversial whether or not the generation of new β-cells indeed occurs in the adult pancreas, a recent report from Heimberg's group provided compelling evidence, by using a cell-lineage tracing technique coupled with a gene-targeting strategy, that regeneration of β-cells in the pancreatic ductal ligation model occurs through Ngn3-expressing progenitor population[33]. It is noteworthy that the expression of CCK2R is markedly upregulated in duct cells before regeneration, in a ductal ligation model[34]. In addition to the activation of Ngn3 by E&G in AR42J cells (Figure 5), 1 week of E&G treatment of *db/db* mice induced a fivefold increase in Ngn3 expression in the pancreas (Figure 7). These findings, together with the identification of small irregular islets in close proximity to ductal structures in E&G-treated animals, further supported our hypothesis that E&G might induce β-cell differentiation from putative progenitors through the activation of Ngn3, as is shown in the ductal ligation model[33]. Another important support to this conclusion comes from the recent report that the same E&G combination therapy expands the β-cell mass in human islets implanted in immunodeficient diabetic mice, possibly from pancreatic duct cells associated with the islets[29].

While it has been reported that the addition of gastrin to various β-cell growth agents (TGF-α, TGF-β, EGF and GLP-1) showed significant therapeutic potential for diabetes[25–27,48], the molecular mechanism underlying the role of gastrin and the mode of collaboration between the two agents is poorly understood. As shown in the present study, Ngn3 induction through p38 MAPK activation and other key molecules, such as MafA and NeuroD1, might be one of the mechanisms of β-cell differentiation induced by gastrin-based therapy.

In conclusion, the results of the present study showed that a short course of combination therapy of exendin-4 and gastrin on prediabetic *db/db* mice preserved β-cell mass, possibly by inducing β-cell differentiation and proliferation. These results suggest that co-activation of GLP-1 receptor and CCK2R in the adult pancreas represents a potentially promising strategy for the induction of new β-cells, thereby compensating for β-cell loss associated with type 2 diabetes.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 | Materials and Methods.
Figure S1 | Biological effects of gastrin on rat insulinoma cell line INS-1.
Table S1 | Antibodies used in this study
Table S2 | Primers used for RT-PCR

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