Marked Expansion of Exocrine and Endocrine Pancreas with Incretin Therapy in Humans with Increased Exocrine Pancreas Dysplasia and the Potential for Glucagon-Producing Neuroendocrine Tumors

Running Title: Human pancreas and incretin therapy

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Abstract

Controversy exists regarding the potential regenerative influences of incretin therapy on pancreatic β cells versus possible adverse pancreatic proliferative effects. Examination of pancreata from age matched organ donors with type 2 diabetes (DM) treated by incretin therapy (n=8) or other therapy (n=12) and non diabetic controls (n=14) reveals a ~40% increased pancreatic mass in DM treated with incretin therapy with both increased exocrine cell proliferation ($p<0.0001$) and dysplasia (increased pancreatic intraepithelia neoplasia, $p<0.01$). Pancreas in DM treated with incretin therapy was notable for α cell hyperplasia and glucagon expressing microadenomas (3/8) and a neuroendocrine tumor. β cell mass was reduced by approximately 60% in those with DM, yet a 6 fold increase was observed in incretin treated subjects although diabetes persists. Endocrine cells co-staining for insulin and glucagon were increased in DM compared to non diabetic controls ($p<0.05$) and markedly further increased by incretin therapy ($p<0.05$). In conclusion, in humans, incretin therapy resulted in a marked expansion of the exocrine and endocrine pancreatic compartments, the former being accompanied by increased proliferation and dysplasia, the latter by α cell hyperplasia with the potential for evolution into neuroendocrine tumors.

Type 2 diabetes (DM) is characterized by defective insulin secretion in the setting of insulin resistance, leading to hyperglycemia. This defect in insulin secretion is accompanied by a deficit in β cell mass. However, the extent and relevance of this β cell deficit has been questioned, in part due to the paucity of human pancreatic studies as well as methodological differences between such efforts (1-3). Therapeutic hope for DM has recently been raised by the introduction of a glucagon like peptide-1 (GLP-1) mimetic class of drugs widely referred to as incretins. Interestingly, beyond their effects on improved metabolic regulation, GLP-1 mimetic therapy was also noted to induce β cell regeneration in rodents; thus portending the remarkable notion that the deficit in β cell mass in DM might be overcome with such agents (4-7). However, given this ability was most evident in the period coincident with the postnatal expansion of β cell mass in rodents, questions arose as to the relevance of this property of GLP-1 in adult humans (8; 9). Moreover, β cell replication was not detected in human islets exposed to high concentrations of GLP-1 *in vitro* (10). In contrast there are concerns that the pro-proliferative actions of GLP-1 might induce deleterious effects on the exocrine pancreas in which the capacity for the pro-proliferative actions of GLP-1 appear to be better retained into adult life (5; 11; 12).

To address this, we analyzed a series of high quality (i.e., transplant grade) human pancreata obtained from brain dead organ donors with and without DM, including a subgroup of the latter subjected to $\geq$ 1 yr of incretin therapy (13). Our goals were to confirm that β cell mass was indeed decreased with DM overall, as well as to establish whether incretin therapy induced an expansion of the endocrine and/or exocrine pancreas.

Research Design and Methods

Study Subjects (Table 1). All pancreata were procured from brain dead organ donors by the JDRF Network for Pancreatic Organ Donors with Diabetes (nPOD) coordinated through the University of Florida in Gainesville, Florida (Table 1) (13). All procedures were in accordance with federal guidelines for organ donation and the University of Florida Institutional Review Board. Pancreata were procured from 20 individuals with DM. These were subdivided into 12 who did not receive GLP-1 drugs (DM) and 8 who received incretin therapy for a year or more, 7 being treated with the DPP-4 inhibitor sitagliptin (Januvia®) and 1 with the GLP-1 mimetic exenatide (Byetta®) (DM-I). Pancreata were also obtained from 14 non diabetic (ND) controls matched by age, sex and BMI with the two DM treatment groups.

Pancreas fixation, embedding and sectioning. nPOD employs a standardized preparation procedure for pancreata recovered from cadaveric organ donors (13). The pancreas is divided into three main regions (head, body and tail) followed by serial transverse sections throughout the medial to lateral axis, allowing for sampling of the entire pancreas organ while maintaining anatomical orientation. As preparation is completed within 2 hours, tissue integrity is maintained. Tissues intended for paraffin blocks are trimmed to no larger than 1.5cm x 1.5cm pieces and fixed in 10% neutral buffered formalin for 24±8 hours. Fixation is terminated by transfer to 70% ethanol and samples are subsequently processed and paraffin embedded. Mounted transverse sections were obtained from the head, body and tail of the paraffin-embedded tissue blocks.

Immunostaining.

In Florida. Formalin-fixed paraffin-embedded sections were stained according to established procedures as previously described (14). In brief, serial sections (4μm) were deparaffinized and incubated with primary antibodies to Ki67 and insulin, or CD3 and glucagon with antibody localization visualized with peroxidase-DAB and alkaline phosphatase-Fast Red polymer systems. Assays included positive and negative controls for each antibody and new lots of reagents were validated to reproduce previous staining intensities using normal human spleen for Ki67 and CD3 and pancreata from donors without diabetes for insulin and glucagon.

In Los Angeles. Sequential paraffin tissue sections from each region of pancreas from each case were stained for 1) Ki67, insulin and Alcian blue by immunohistochemistry and 2) Ki67 and glucagon by immunohistochemistry. In addition, a section of pancreas from each of the DM cases treated with incretin therapy and a section from a subset of DM not treated with incretin therapy (5 cases) and non-diabetic cases (6 cases) were stained for insulin and glucagon by immunofluorescence, and additional sections for glucagon, insulin, cytokeratin and DAPI.

Briefly, the Cytokeratin, Insulin and Glucagon slides were stained as follows: CK-19 (mouse 1:50 4°C overnight, Dako, Carpinteria , CA); Glucagon (rabbit 1:1000 4°C overnight, Immunostar Inc, Hudson, WI); Insulin (guinea pig 1:100 4°C overnight, Invitrogen, Grand Isle, NY). FITC, Cy3 and Cy5 followed incubation of each primary antibody respectively (1:100, Jackson Laboratories, Sacramento, CA). Slides were coverslipped with Vectashield with DAPI (Vector Laboratories, Burlingame, CA).

Morphometric analysis.

In Florida. Stained slides were scanned to create whole digital slide images with an Aperio ScanScope CS (Aperio Technologies Inc., Vista, CA) and Spectrum Plus version 11 at

20x magnification. Each image was reviewed and annotated using the Aperio ImageScope image viewing program. Regions with edge artifact or non-specific staining were excluded from analysis. The Spectrum colocalization algorithm was used to estimate the proportion of insulin and glucagon stained area compared to total tissue area defined by the hematoxylin counterstain. Software parameter settings were optimized for detection of both hematoxylin and fast red chromogen. Data were averaged within regions then averaged between all regions for a given donor pancreas. Cyto-nuclear IHC quantification software (Indica Labs, NM) was used to quantify total cell numbers per pancreas section and total cell replication rates using Ki67 nuclear immunopositivity. Input parameters were set using an ImageScope plugin tool that calculates optical densities of the underlying structures in RGB OD values. Total cell counts were estimated from nuclei stained with hematoxylin. Nuclei co-stained with Ki67 were counted and expressed as a percentage of total.

Full cross-sections of the pancreas head, body and tail were evaluated for pancreatic intraepithelial neoplasia (PanIN) by a gastrointestinal pathologist blinded to clinical information and using the established consensus classification system for these precursor lesions (15). The total number of PanIN lesions and their grade were determined per lobular unit and scored as the highest grade lesion within that lobule. The number of PanINs per pancreas section was then computed per unit area of pancreas ($mm^2 x10^3$).

In Los Angeles.

Whole sections of pancreas stained for insulin, Ki-67 and Alcian Blue and hematoxylin counter stain were digitally scanned using Aperio ScanScope (Aperio Technologies Inc., Vista, CA). Analysis was performed using Aperio ImageScope version 11.0.2.725. With this software, the total area of each tissue section was determined.

Using the insulin, Ki-67 and Alcian blue stained sections, 100 islets were analyzed per section using an Olympus CX41 microscope (Olympus America, Center Valley, PA) to determine the frequency of Ki67 in the β cells of islets and in the non- β cell compartment of those islets. Similarly, using the glucagon and Ki-67 stained sections, 100 islets per section were analyzed to determine the frequency of Ki67 in the α cells of islets and in the non- α cell compartment of those islets. The mean β and α cell diameter was determined in each individual using the insulin and glucagon immunostained sections from the pancreas body as previously described (16). A total of 475 α cells and 475 β cells were evaluated.

To determine the percentage of β and α cells within pancreatic ducts, the insulin-Ki67-Alcian blue stained slides and the Glucagon-Ki67-hematoxylin slides were used. The total number of duct cells per pancreatic section was counted manually, as were the total number of cells within the ducts that stained for insulin or glucagon respectively. The results are expressed as the percentage of duct cells positive for insulin or glucagon.

Using the sections stained by immunofluorescence for insulin, glucagon and DAPI, a minimum of 30 islets per section (range 31 to 45, mean 38.0±1.0) were randomly selected and imaged at 20x magnification and stored using a Leica DM6000 fluorescent microscope (Leica Microsystems Inc., Deerfield, IL) connected to a Macintosh computer loaded with Openlab software (Improvision, Lexington, MA). Each islet was analyzed to determine number of β cells, number of α cells and number of cells co-staining for insulin and glucagon.

Confocal microscopy was performed where cells co-staining for insulin and glucagon were detected for confirmation that insulin and glucagon granules were indeed both present in the same cells. Imaging was performed using a scanning confocal microscope equipped with argon, green and red helium-neon lasers (Leica). Images were acquired by sequential scanning

using a ×20 objective and the appropriate filter combination. Z sections were captured with a 0.25 μm step size.

Statistical analysis.

Statistical analysis was performed using Student's t test (GraphPad Prism version 5.0, GraphPad Software Inc., La Jolla, CA). Data in graphs and tables are presented as means ± SEM. Findings were assumed statistically significant at the $P<0.05$.

Results

Pancreatic mass was increased ($p<0.05$) approximately 40 percent in DM patients treated with incretin therapy compared to that in individuals with DM not treated with such agents (Figure 1A). The pancreatic fractional insulin area was approximately 60 percent reduced in DM patients not treated with incretin therapy compared to non-diabetic controls (0.34±0.10 *vs* 0.79±0.10%; $p<0.001$) (Figure 1B). In contrast, the pancreatic fractional insulin area was approximately 5-fold increased in DM patients treated with incretin when compared to individuals not treated with incretin therapy (1.60±0.41 *vs* 0.34±0.10 %, $p<0.0001$).

The β cell mass, computed from the product of the pancreatic fractional insulin positive area and pancreatic mass, was 55% decreased in DM patients not on incretin therapy in comparison to non-diabetic controls (Figure 1D; 0.29±0.08 *vs* 0.60±0.10G; $p<0.05$). In contrast, β cell mass was increased 6-fold in incretin treated DM patients (1.81±0.56 *vs* 0.29±0.08G, $p<0.01$). Moreover β cell mass was 3-fold greater in individuals with DM treated with incretins in comparison to non diabetic controls (1.81±0.56 *vs* 0.60±0.10 G, $p<0.05$). The 6-fold increase in β cell mass in DM with incretin therapy was almost all due to increased β cell numbers rather

than β cell size although there was a 3% increase in β cell size with incretin therapy (8.9±0.1 vs 8.6±0.1μm, DM vs DM-1, $p<0.05$, Figure 1C). The pancreatic fractional area immunostained for glucagon was comparable between individuals with DM not subject to incretin therapy and non-diabetic controls (0.57±0.12 *vs* 0.52±0.08%, p=NS, Figure 1E). However, the pancreatic fractional area immunostained for glucagon was markedly increased in individuals with DM treated with incretin therapy versus DM on other therapy (1.65±0.39 *vs* 0.57±0.12%, $p<0.0001$), as well as compared to non-diabetic controls (1.65±0.39 *vs* 0.52±0.08%, $p<0.0001$). The pattern of the calculated glucagon mass followed that of the pancreatic fractional area, being comparable in individuals with DM not treated with incretin therapy and controls, but 5 fold increased in DM individuals treated with incretin therapy compared to DM not treated by incretin therapy (2.08±0.75 *vs* 0.45±0.10 G, DM-I vs DM, $p<0.01$, Figure 1G). The marked increase in α cell mass, like that of β cell mass, was almost completely due to an increase in the number of α cells although again there was a 4% increase in α cell size (8.9±0.1 vs 8.6 ±0.1μm, DM-I vs DM, $p<0.01$, Figure 1F).

There were two immediately striking findings on inspection of the pancreatic sections of the cases of DM treated by incretin therapy; a subset of enlarged and often eccentrically shaped islets, as well as increased numbers of endocrine cells in association with duct structures (Figure 2, 3, 4). The impression of a subset of enlarged islets was confirmed quantitatively; the 12 largest islets per section in the incretin treated DM cases being almost twice the cross sectional area of those in the DM subjects not treated by incretins (82,270±10,330 *vs* 44,770±6,986 μM^2, $p<0.01$). While examples of insulin immunoreactive cells related to ductal structures could be found in individuals from all three groups, the percentage of such cells within ducts was not increased in DM treated with versus DM without incretin therapy (0.30±0.09 *vs* 0.27±0.07%,

p=NS, Figure 3, 4). However, glucagon immunoreactive cells were frequently found in long linear groups or solid nests of cells either within the duct itself or in the immediate periductal location. In addition, these glucagon positive cells also formed intraductal luminal projections as previously described in chronic pancreatitis (17) (Figure 2A). There were also regions with multiple small ducts that had glucagon expressing tubular and islet like structures directly contiguous to the ducts. Of note, there were cells expressing both cytokeratin and glucagon at the interface of these α cell tubular projections from ducts (Figure 2 F-I). The percentage of cells immunoreactive for glucagon in ducts was increased in DM with prior incretin therapy versus DM with no incretin therapy (2.8±0.9 *vs* 0.5±0.2%, p=<0.05) (Figure 3E). Thus while the exuberant increase in glucagon immunoreactive cells with incretin treatment were frequently observed in the periductal areas, the increased numbers of insulin immunoreactive cells with incretin therapy tended to be most abundant more remote from these periductal endocrine complexes (Figure 3B,D). Pancreas from the single individual treated by Exenatide also revealed exuberant α cell hyperplasia (Figure 4).

In one individual with DM treated with Sitagliptin (nPOD case 6185), a 1.5 cm α cell/glucagon producing neuroendocrine tumor (Grade 1, World Health Organization, 2010) not appreciated in life was identified in the body of the pancreas after resection at brain death (Figure 5). Glucagon-producing microadenomas were also detected in the same case (6185) and two other incretin treated cases (6157 and 6206) (Supplemental Figure 3), while hyperplastic islets with predominant glucagon staining were noted in 7 of 8 of the incretin treated cases. No neuroendocrine tumors or glucagon-producing microadenomas were detected in non diabetic controls or DM not treated with incretin therapy.

Inspection of pancreatic sections immunostained with either insulin or glucagon from individuals with DM treated by incretin therapy gave the impression that numerous cells within these islets were immunoreactive for both hormones. When quantified in sections double immunostained for glucagon and insulin by confocal microscopy for this purpose (Figure 6, Supplementary Figures 1,2), the percentage of insulin positive cells in incretin treated individuals that were also glucagon immunoreactive were indeed markedly increased when compared to those with DM not treated with incretin therapy (16.8±5.0 *vs* 3.2±1.4%, $p<0.05$). Interestingly there was also an increase in double immunoreactive positive cells in individuals with DM not treated with incretin therapy when compared to non-diabetic controls (3.2±1.4 *vs* 0.4±0.1%, $p<0.05$). As in prior human studies (2), the frequency of Ki67 positive nuclei in islet endocrine cells was extremely rare (all less than 0.01 cells per islet section), with no significant differences in this very low frequency of β or α cell replication between any of the three groups studied.

The increased pancreatic mass in DM induced by incretin therapy was accompanied by increased whole pancreas cell proliferation (0.25±0.03 Vs 0.12±0.01%, DM-I vs DM, $p<0.0001$) and an increase in the presence of pancreatic intraepithelial neoplasia (PanINs) (11.9±2.6 vs 4.9±1.7, DM-I vs DM, PanINs/mm^2 x 10^3, $p<0.01$) (Figure 7). PanIN lesions were also not infrequently detected in or close to these areas with ductular α cell complexes (Figure 7). Inspection of pancreas sections in incretin treated individuals revealed small foci of increased Ki67 immunostaining in and around ducts and sometimes in areas of exocrine dysplasia (Supplementary Figure 4). Given the focal and often dysplastic nature of these areas of increased cell replication, it was not possible to attribute the increased replication to a particular tissue compartment of pancreas (for example ducts, acinar cells).

Discussion

In this study we took advantage of the unique resource of pancreata procured by the JDRF nPOD program (13) to reevaluate the question of β cell mass in DM and in addition, to address the potential actions of the incretin drugs on the endocrine and exocrine compartments. In individuals with DM not using incretin drugs, we report a 60 percent deficit in β cell mass; but unchanged α cell mass. Perhaps not surprisingly, given methodological differences, previous reports suggest a wide range of change in β and α cell mass (or pancreatic fractional area) in DM (1-3; 18-20).

A striking finding in the present studies is the marked expansion of the exocrine and endocrine compartments of the pancreas with incretin therapy. The DPP-4 inhibitor Sitagliptin acts to increase endogenous GLP-1 levels (21). It is not known whether the actions of Sitagliptin reported here were mediated by increased circulating GLP-1 levels, increased GLP-1 levels produced by the newly formed α cells acting in a paracrine manner, or due to the actions of signaling peptides other than GLP-1 that are degraded by DPP-4. The one individual treated by Exenatide available to us here showed a comparable pattern of changes to those observed in the Sitagliptin individuals (Figure 4), implying that a direct action of GLP-1 is likely involved. However, to fully address this question, it will be important to obtain more pancreata from individuals who have been treated with GLP-1 mimetic therapy.

The pancreatic mass of the individuals with DM treated with incretins was increased by 40 percent in comparison to diabetics not treated with incretin therapy, consistent with the prior rodent studies that revealed proliferative actions of GLP-1 on the exocrine pancreas now extended here to humans (5; 12). Also, of particular concern, incretin therapy was associated with an increase in pancreatic dysplastic PanIN lesions, consistent with the prior finding that

GLP-1 receptors are expressed not only in the human exocrine pancreas but also in PanINs, and that GLP-1 induces proliferative signaling in human pancreatic duct epithelia cells (12). Moreover, GLP-1 accelerated mPanIN formation in the $Kras^{G12D}$ mouse model (12). Of interest, in incretin treated DM, PanINs and ductal endocrine complexes were often seen in close proximity (Figure 7), which could suggest a common underlying dysplastic process that perhaps adds insight into the admixture of endocrine cells often noted within pancreatic adenocarcinoma (22). The increased cellular proliferation observed in the whole pancreas sections of incretin treated T2D patients (Figure 7E) could theoretically be due to a contribution of intrapancreatic GLP-1 released by newly formed α cells. It is unlikely that brain dead individuals in an intensive care unit setting for the week prior to organ procurement would be treated by incretin therapy. To more precisely elucidate the direct action of those therapies on proliferation in human pancreas, and to investigate the cellular subcompartments in which they induce this action it will also be important to seek opportunities to obtain human pancreas from individuals who had incretin therapy up until pancreas was obtained, perhaps via surgery.

The marked α cell hyperplasia, glucagon expressing microadenomas and glucagon expressing neuroendocrine tumor noted in individuals with DM treated with incretin therapy are also of concern. These findings reproduce the α cell hyperplasia, abnormal α cell distribution and predisposition to glucagon expressing neuroendocrine tumors previously reported with suppressed glucagon secretion or signaling (23-25). It is of note that a consistent action of incretin therapy is to suppress glucagon secretion. As in prior reports of decreased glucagon secretion or action, the expansion in α cell mass in incretin treated DM cases was prominently distributed as tubular outgrowths of glucagon expressing cells from small duct-like structures, within the lining of (or surrounding) larger ducts occasionally forming intraductal projections

within the lumen. The latter possibly contribute to the reported increased incidence of pancreatitis in incretin treated patients (11). Pancreatitis has previously been reported in humans with unexplained α cell hyperplasia (26) and intraductal endocrine cell projections have been reported previously in chronic pancreatitis (17). Therefore incretin therapy induced intra and periductal α cell proliferations are now added to incretin therapy induced expansion of PanIN lesions as plausible mechanistic links to induction of pancreatitis through obstruction of pancreatic enzymes outflow.

The source of new α cells in prior studies of impaired glucagon secretion or signaling was deemed most likely as arising from progenitor cells since no increase in α cell replication was detected (23-25). We were also unable to detect any increase in α cell replication in incretin treated DM to account for α cell hyperplasia. Although again it should be noted that the study subjects were likely not exposed to study drugs immediately prior to pancreas procurement, a prior study suggests Sitagliptin therapy suppresses rather than enhances α cell replication (27). Given that α cells are epigenetically relatively unstable (28), and that most glucagonomas display malignant behavior, the present finding calls into question the safety of long-term suppression of glucagon secretion or action. Moreover, since the standard of care of a pancreatic neuroendocrine tumor, because of the risk of conversion to malignancy, even if benign, is surgical resection, patients exposed to incretin therapy would seem to be at increased risk of requiring pancreatic surgery.

The present finding of an increased pancreatic mass, increased PanIN lesions and endocrine proliferations encircling and sometimes encroaching on pancreatic ducts in response to GLP-1 mimetic therapy does add to concerns already raised regarding the potential adverse actions of GLP-1 mimetic therapy to induce pancreatitis and accelerate pancreatic dysplasia (11).

Prior reports of potentially concerning pancreas changes with incretin therapy were confined to studies of rodent pancreas but are now here extended to humans with the added concern of the risk of neuroendocrine tumors. Since newly formed α cells in response to impaired glucagon secretion have been shown to secrete high levels of GLP-1 (23-25), the local GLP-1 concentrations in pancreas in patients treated with DPP-4 inhibitors may be very high, particularly where these cells are abundant in and around dysplastic ducts. This may account for the observed increase in pancreatitis and pancreatic tumors registered with the FDA adverse event reporting system for GLP-1 mimetics and DPP-4 inhibitors, in contrast to the signal for thyroid tumors being restricted to GLP-1 mimetics (11). It also may explain the expansion of the exocrine pancreas in glucagon receptor null mice (and mutant human), exocrine proliferation being noted in both as well as high GLP-1 release by newly formed α cells (23-25).

At first sight, the increase in β cell mass with incretin therapy in DM would appear to be an exciting finding in relation to the potential for disease reversal. Unfortunately, these insulin-expressing cells are presumably not functionally mature since the incretin treated individuals still had diabetes. It will be important to establish the molecular signature of insulin expressing cells in future studies. The marked expansion of the β cell mass in individuals with DM treated with Sitagliptin or Exenatide for a year or more is in contrast to the findings of the effects of these drugs on β cell function in DM (29; 30). While β cell function benefits to some extent in patients with DM with any therapy that lowers blood glucose values (31; 32), no data to date suggests that there is a disease modifying action of incretin therapy in DM over that of any other glucose lowering agent with regard to recovery of β cell function (29; 30). It is plausible that GLP-1 directly induced proliferation of β cells as has been reported in animal studies (4; 5). However these studies were in juvenile rodents, and GLP-1 did not induce proliferation in human islets *in*

vitro (10). Also β cell replication did not differ between groups in this study. However, as stated previously, as incretin therapy was likely not administered during the last week of life in the present donors, it is not possible to exclude incretin induced β cell replication from this study. Likewise, it is conceivable that the increase in β cell number in incretin treated DM may be due in part to GLP-1 mediated inhibition of β cell apoptosis.

Another striking finding in the islets of the incretin treated individuals is a high proportion of cells that expressed both insulin and glucagon. This has been described in the newly forming endocrine pancreas at week 20 of gestation in humans (33), and thus might be an indication of newly formed immature endocrine cells from presumptive progenitors. Alternatively, lineage tracing studies in diabetic mice have established that α cells have the capacity to transdifferentiate towards a β cell phenotype (34). A third possible source of these double hormone expressing cells could be β cells that have partially transdifferentiated towards an α cell fate. The epigenetic basis of this inter-conversion has been established (35).

In summary, we confirm that in humans with DM, β cell mass is deficient while α cell mass is no different to that in non-diabetic individuals. We note that both the exocrine and endocrine pancreas is markedly enlarged in individuals with DM treated with incretin therapy with increased exocrine cell proliferation. The α cell hyperplasia and neuroendocrine tumor and microadenoma formation is consistent with chronic inhibition of glucagon secretion by GLP-1. These findings lend additional weight to concerns regarding the effects of long term GLP-1 related therapy with respect to both unintended proliferative actions on the exocrine pancreas and now also a possible increased risk of neuroendocrine tumors. In addition to the surveillance previously recommended for the potential association of GLP-1 based therapy and pancreatic cancer risk, the current data imply that surveillance for a possible increased risk of pancreatic

neuroendocrine tumors is warranted. On the other hand, the finding that there is a capacity to form large numbers of insulin expressing cells in adult humans offers an impetus to the hopes of the goal of inducing β cell regeneration in either type 1 or 2 diabetes. It is clear that a priority will be to establish the pathway by which these cells are formed and how to favor an endocrine versus exocrine cell lineage and, within the latter, a β cell lineage. Moreover, further investigation on how to foster the maturation of the newly formed β cells to function is of vital therapeutic importance.

Author Contributions

Alexandra E. Butler and Martha Campbell-Thompson independently evaluated the morphometric analysis of pancreas. Martha Campbell-Thompson and Mark Atkinson supervised procurement of the pancreata by the nPOD program, and the immunostaining procedures in Florida and participated in interpretation of the data. Peter C. Butler and Tatyana Gurlo supervised the immunostaining procedures in Los Angeles and participated in interpretation of the data. Dr. David Dawson performed pathologic evaluation of pancreas sections. Alexandra E. Butler, Peter C. Butler and Mark Atkinson contributed to writing the manuscript and preparation of the figures. Peter C. Butler is the guarantor of the manuscript and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Tables

Table 1

DM INCRETIN

	Age [years]	Duration of DM [years]	Sex	BMI	Treatments	Causes of Death
6157	74	1	F	39	Januvia	ICH/Stroke
6185	46	15	M	41	Januvia, Metformin;	Anoxia
6186	68	5	M	21	Januvia , Metformin	ICH/Stroke
6189	49	26	F	36	Byetta, Metformin, Glipazide	Stroke
6199	53	20	M	30	Januvia, Insulin pen	ICH/Stroke
6194	47	13	M	24	Humulin, Novolog, Januvia	ICH/Stroke
6203	68	5	M	33	Januvia, Metformin	Stroke
6206	59	10	M	42	Januvia, Metformin	Stroke
Mean	**58**	**12**		**33**		
SEM	**4**	**3**		**3**		

DM

	Age [years]	Duration of DM [years]	Sex	BMI	Treatment	Causes of Death
6028	33	17	M	30	Insulin	Gunshot wound to head
6059	18	0.3	F	39	None	Cardiovascular

6108	57	2	M	30	Metformin	ICH/Stroke
6110	20	0.2	F	40	None	ICH/Stroke, DKA
6109	48	-	F	33	None	ICH/Stroke, DKA
6114	42	2	M	31	Metformin, noncompliant	Asphyxiation
6124	62	3	M	34	Metformin	ICH/Stroke
6127	44	10	F	30	Insulin	ICH/Stroke
6133	45	20	F	40	Insulin	Cardiovascular
6139	37	1.5	F	45	None	Seizure
6142	29	14	F	34	None	Bacterial Meningitis
6149	39	20	F	29	Insulin	ICH/Stroke
Mean	**40**	**8**		**35**		
SEM	**4**	**3**		**2**		

NON DIABETIC

	Age [years]	**Duration of DM [years]**	**Sex**	**BMI**	**Treatment**	**Causes of Death**
6009	45		M	31		Anoxia
6015	39		F	32		Anoxia
6012	64		F	31		Cerebrovascular/Stroke
6016	42		M	31		Cerebrovascular/Stroke
6019	68		F	24		Head Trauma
6020	60		M	30		Cerebrovascular/Stroke

6022	75	M	31	Cerebrovascular/Stroke
6034	32	F	25	Head Trauma
6060	24	M	33	Head Trauma
6097	43	F	36	Cerebrovascular/Stroke
6099	14	M	30	Head Trauma
6102	45	F	35	Cerebrovascular/Stroke
6158	40	M	30	Head Trauma
6165	45	F	25	Cerebrovascular/Stroke
Mean	**45**		**30**	
SEM	**5**		**1**	

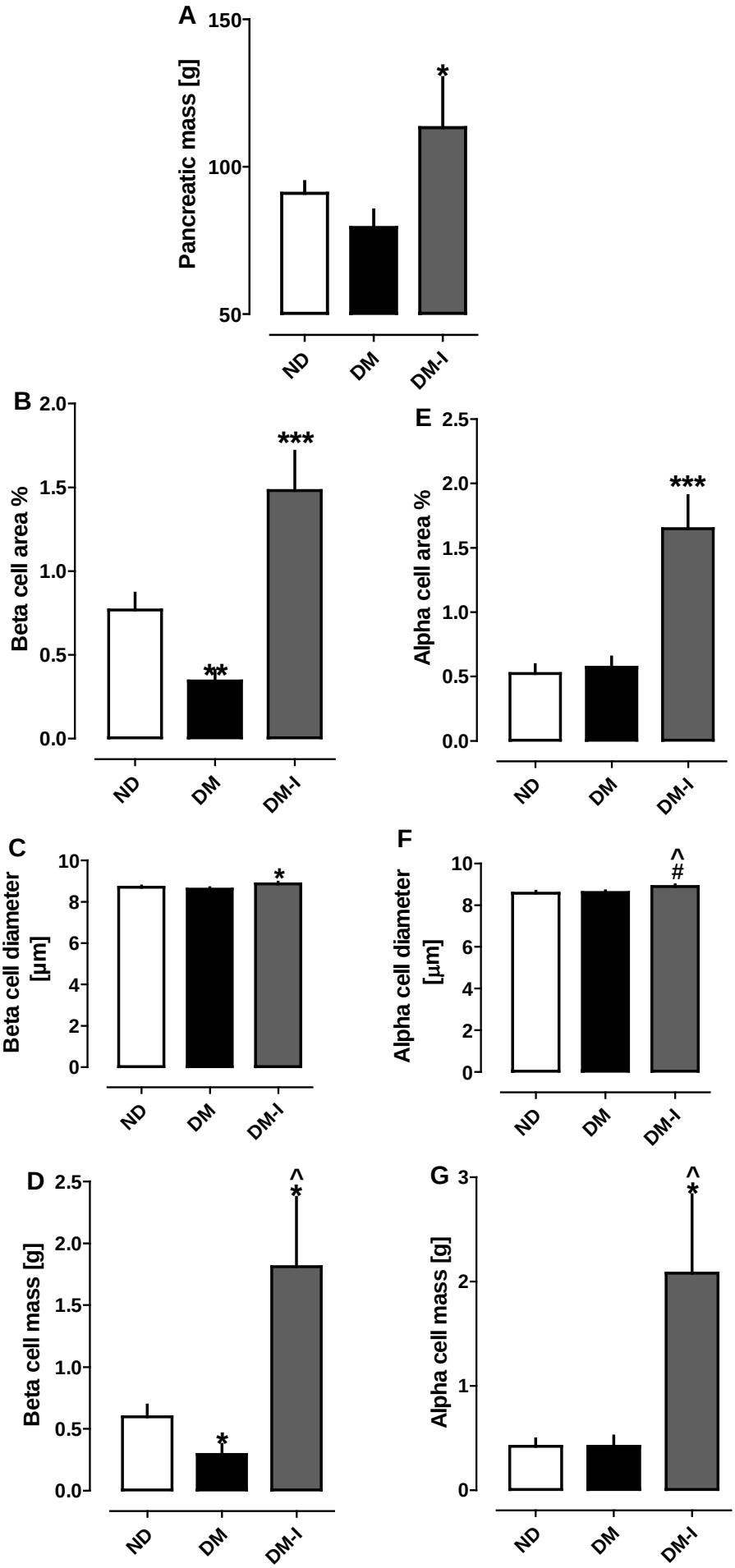

A
Pancreatic mass [g]
150
100
50
*
ND
DM
DM-I
B
Beta cell area %
2.0
1.5
1.0
0.5
0.0
***
**
ND
DM
DM-I
E
Alpha cell area %
2.5
2.0
1.5
1.0
0.5
0.0
***
ND
DM
DM-I
C
Beta cell diameter [µm]
10
8
6
4
2
0
*
ND
DM
DM-I
F
Alpha cell diameter [µm]
10
8
6
4
2
0
^
#
ND
DM
DM-I
D
Beta cell mass [g]
2.5
2.0
1.5
1.0
0.5
0.0
^
*
*
ND
DM
DM-I
G
Alpha cell mass [g]
3
2
1
0
^
*
ND
DM
DM-I

Figure 1. Pancreas, β cell and α cell mass.

Mean pancreatic weight (A) *p<0.05 DM-I vs DM; pancreatic fractional insulin area (B) *** p<0.0001 DM-I vs DM, ** p<0.001 DM vs ND; β cell diameter (C) *p<0.05 DM-I vs DM; β cell mass (D) ^ p<0.01 DM-I vs DM, *p<0.05 DM-I vs ND, *p<0.05 DM vs ND; pancreatic fractional glucagon area (E) ***p<0.0001 DM-I vs DM and ND; α cell diameter (F) ^ p<0.01 DM-I vs DM, #p<0.005 DM-I vs ND; and α cell mass (G) ^ p<0.01 DM-I vs DM; *p<0.05 DM-I vs ND; in non diabetics (ND), type 2 diabetes without (DM) and with incretin therapy (DM-I). Pancreatic weight was 40% increased in DM-I compared to DM (P<0.05). β cell mass was decreased in DM compared to ND but ~6-fold increased in DM-I compared to DM. α cell mass was comparable in DM and ND but was again ~5-fold increased in DM-I compared to DM. The increase in β and α cell mass with incretin treatment was predominantly due to endocrine hyperplasia rather than hypertrophy.

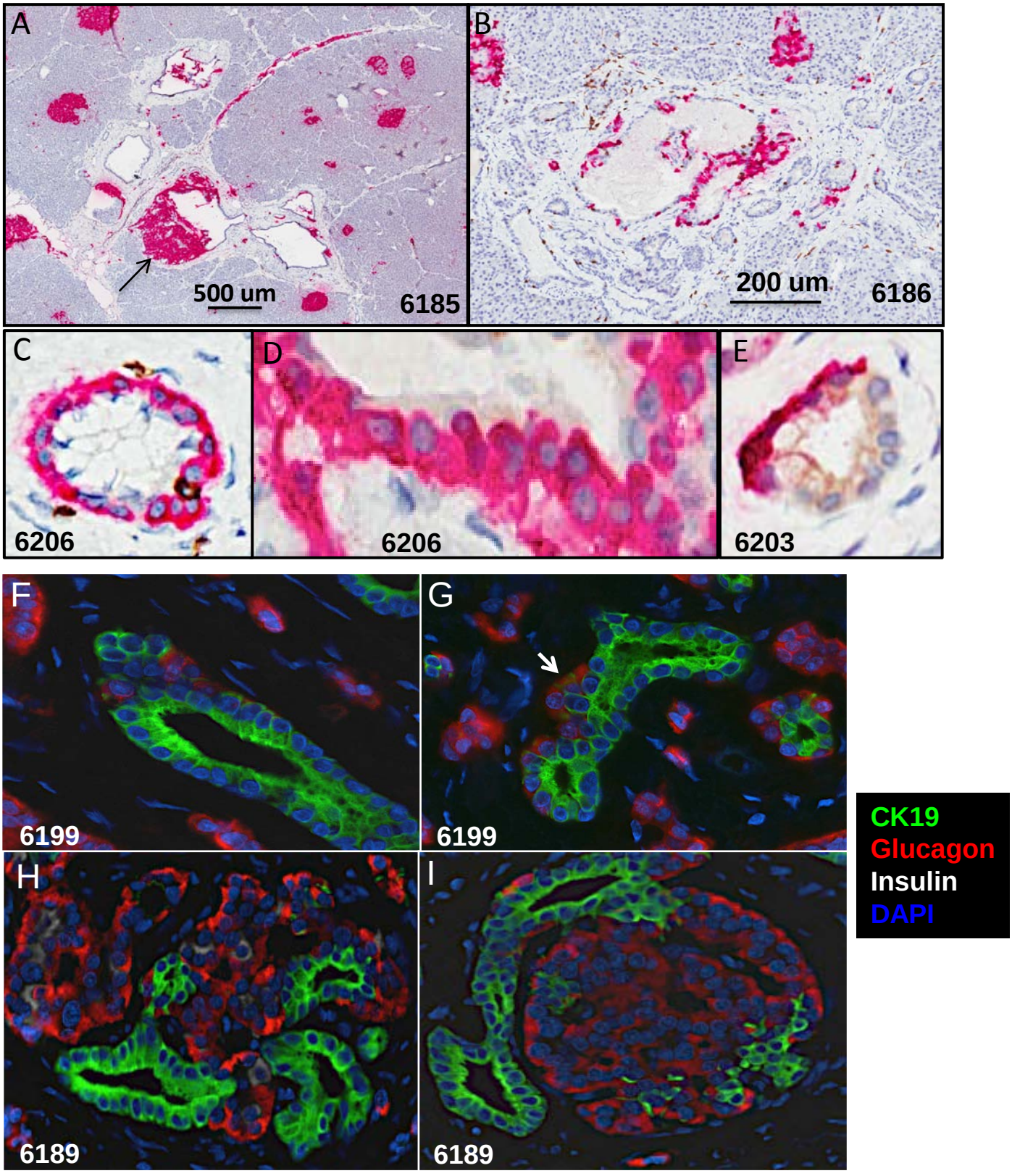
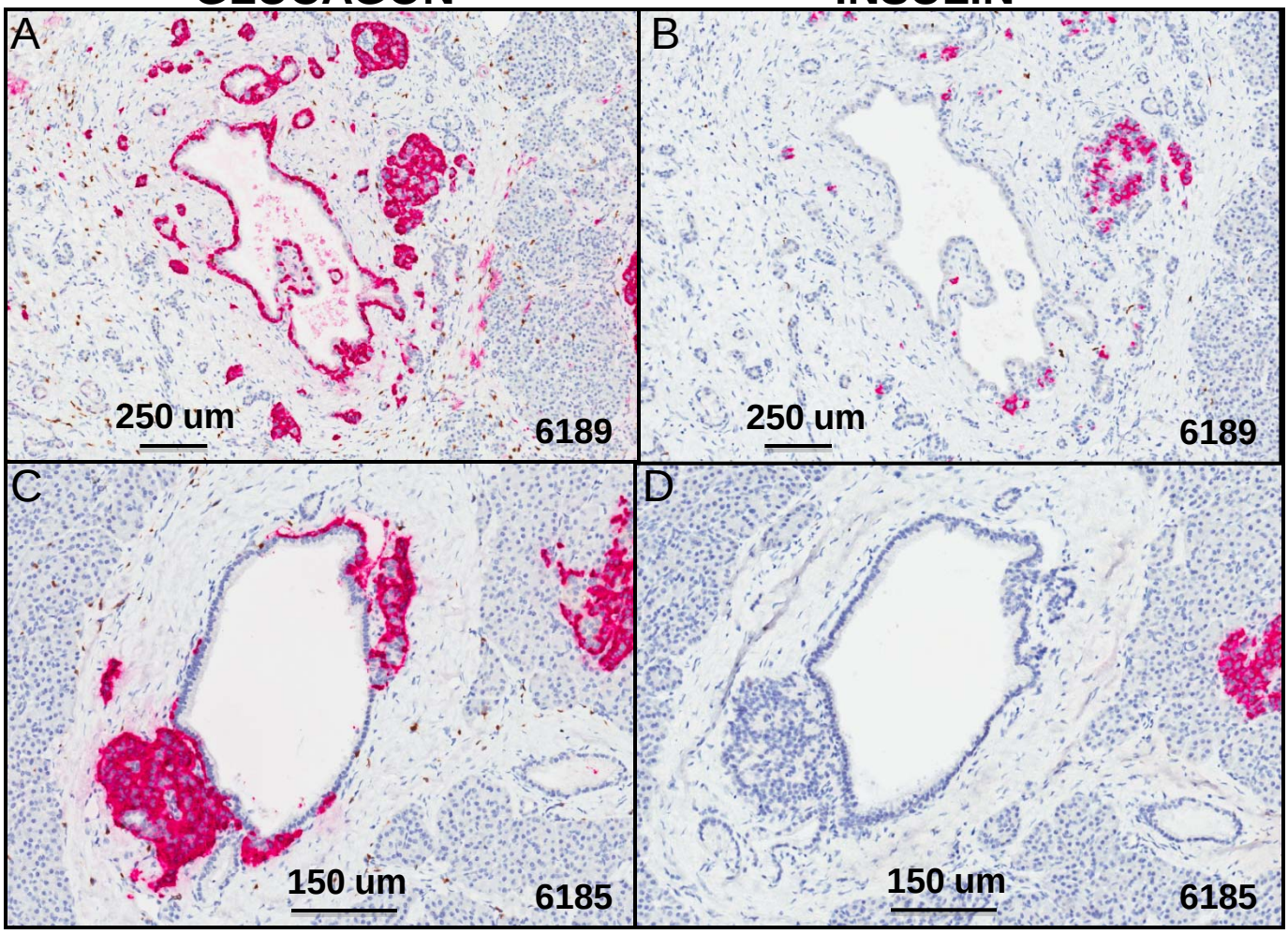
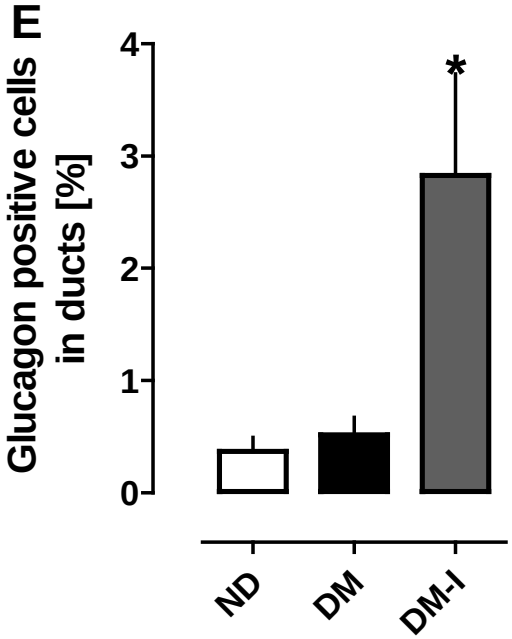

GLUCAGON
A
500 um
6185
B
200 um
6186
C
6206
D
6206
E
6203
F
6199
G
6199
H
6189
I
6189
CK19
Glucagon
Insulin
DAPI

Figure 2. Glucagon immunohistochemistry in pancreas in type 2 diabetes after incretin therapy.

A-E Sections of pancreas from cases with type 2 diabetes after incretin therapy (6185 Sitagliptin, 6186 Sitagliptin, 6206 Sitagliptin, 6203 Sitagliptin) immunostained for glucagon (pink) with hematoxylin counterstain. Exuberant expansion of glucagon immunoreactive cells are seen as enlarged eccentrically shaped islets, as well as nodular and linear aggregates of cells intimately associated with ducts and demonstrating variable extension into duct lumens (arrow). C-E higher power images to show glucagon immunoreactivity in cells lining ducts. F-I Pancreas sections from cases of type 2 diabetes after incretin therapy (6199 Sitagliptin, 6189 Exenatide) showing immunofluorescent co-staining for cytokeratin (green), glucagon (red) and DAPI nuclear counterstain (blue). Glucagon expressing cells are present within and adjacent to keratin-positive duct structures. Arrow in G indicates a cell co-staining for cytokeratin and glucagon.


GLUCAGON
INSULIN
A
B
C
D
250 um
6189
250 um
6189
150 um
6185
150 um
6185


E
Glucagon positive cells in ducts [%]
4
3
2
1
0
*
ND
DM
DM-I

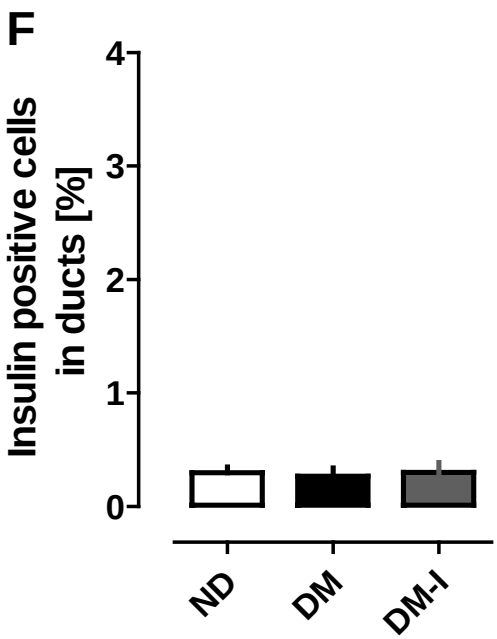

F
Insulin positive cells in ducts [%]
4
3
2
1
0
ND
DM
DM-I

Figure 3. Distribution of α and β cells in relation to ducts following incretin therapy in type 2 diabetes.

(A-D) Serial adjacent sections of pancreas from DM cases after incretin therapy (6189 Exenatide, 6185 Sitagliptin) were immunostained for (A,C) glucagon or (B,D) insulin with hematoxylin counterstain. These serial sections indicate that the exuberant endocrine growth associated with ducts is predominantly comprised of glucagon immunoreactive cells. (E-F) The percentage of glucagon positive cells in ducts (E) and insulin positive cells in ducts (F). The percentage of glucagon positive cells in ducts was increased in the DM-I group when compared with both the DM and ND groups ($p<0.05$). In contrast, the percentage of insulin positive cells in ducts was unchanged in the DM-I group.

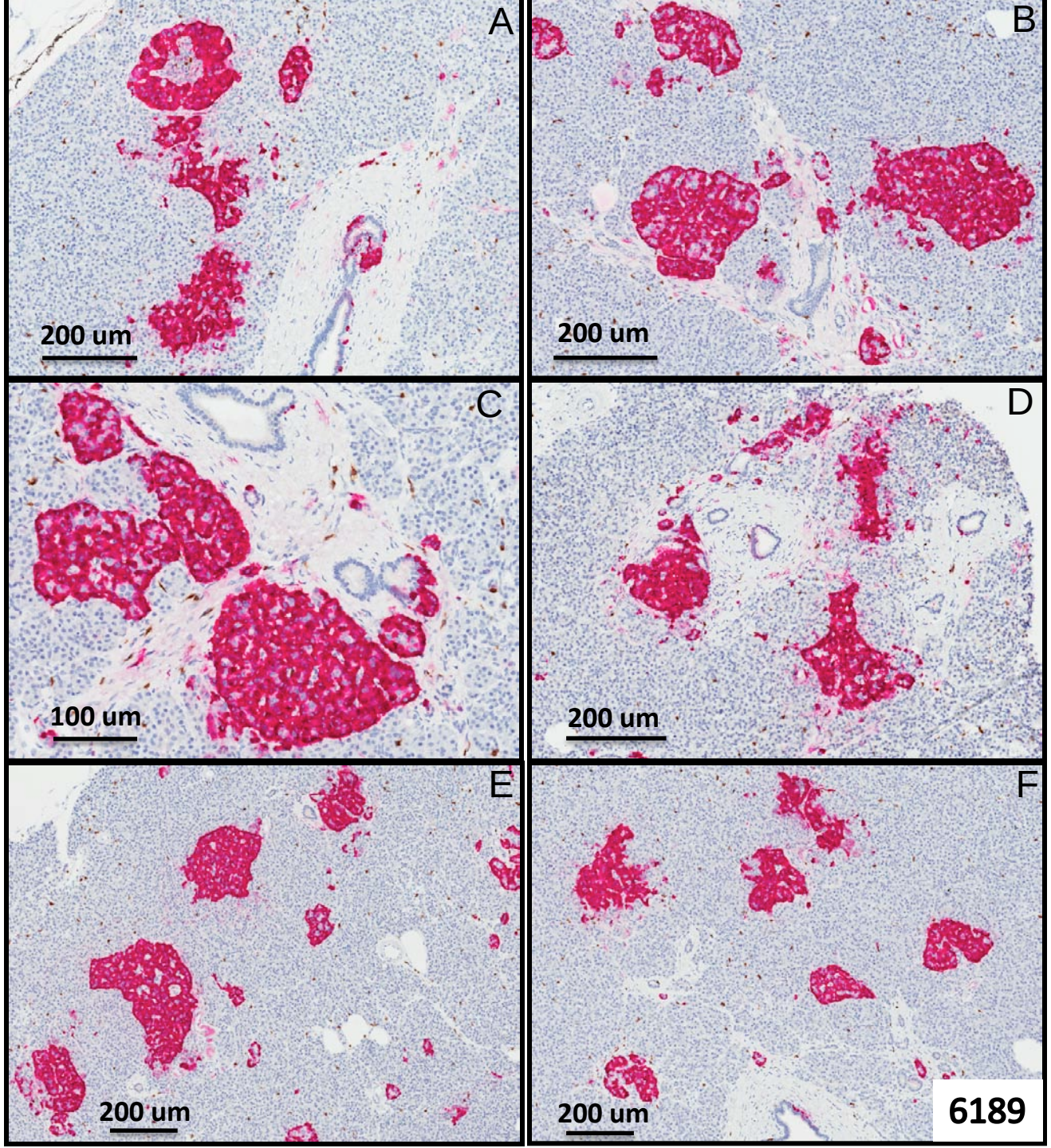

A
200 um
B
200 um
C
100 um
D
200 um
E
200 um
F
200 um
6189

Figure 4. Evidence for a direct role of GLP-1 mimetic action in α cell hyperplasia.

Images of pancreatic sections (A-F) are provided to illustrate a similar pattern of α cell hyperplasia in the donor treated with Exenatide (6189) as the Sitagliptin treated cases, implying a role for GLP-1 action independent of DDP-4 inhibition in α cell hyperplasia. Enlarged and often eccentrically shaped islets are apparent, as well as increased numbers of glucagon immunoreactive cells associated with and surrounding ductal structures.

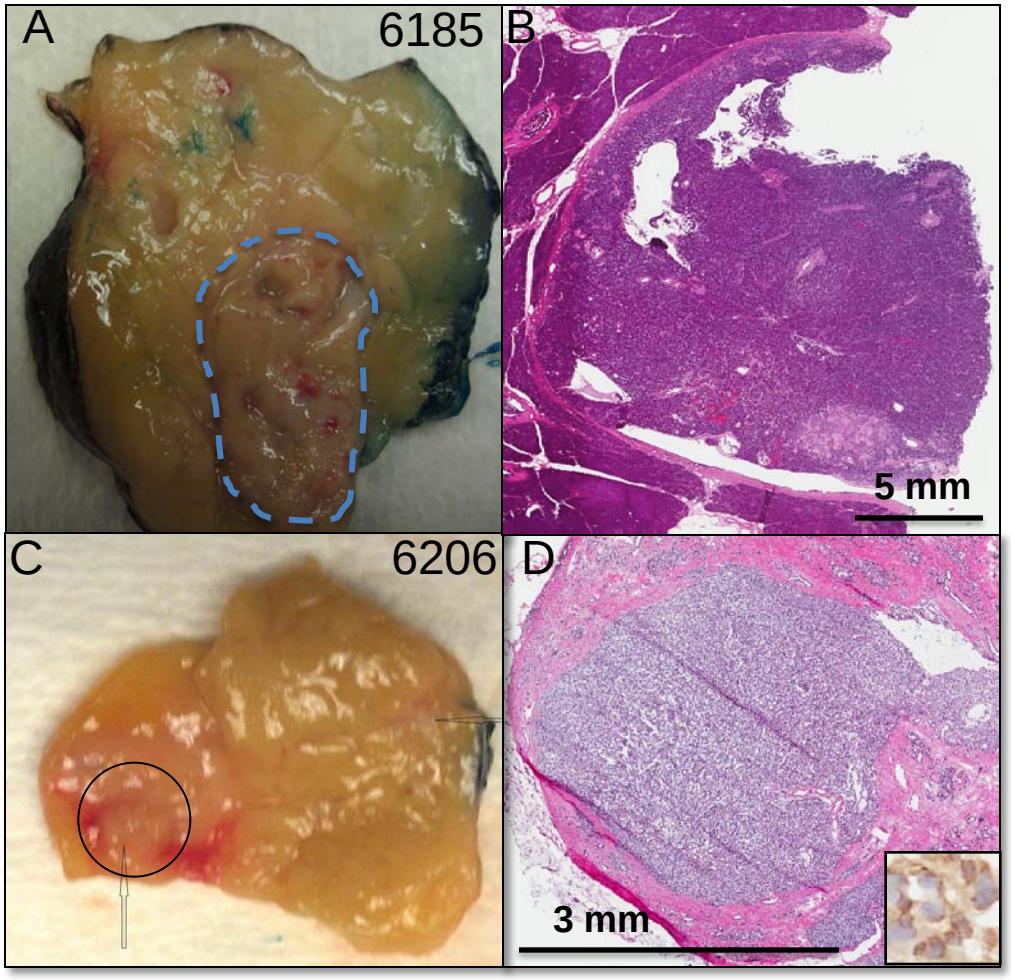

A
6185
B
5 mm
C
6206
D
3 mm

Figure 5. Pancreatic glucagon expressing neuroendocrine tumor and microadenoma.

(A) Grossly visible lesion and (B) corresponding H&E-stained section of the clinically undetected glucagon expressing neuroendocrine tumor in the pancreas of nPOD case 6185, type 2 diabetes after prior Sitagliptin therapy. (C) Gross specimen and (D) corresponding H&E-stained section of a glucagon expressing microadenoma in case nPOD case 6206, type 2 diabetes after Sitagliptin therapy. Inset shows high power view of representative cells stained for glucagon by immunohistochemistry. (See supplementary figure 3 for additional image of microadenoma in case 6206).

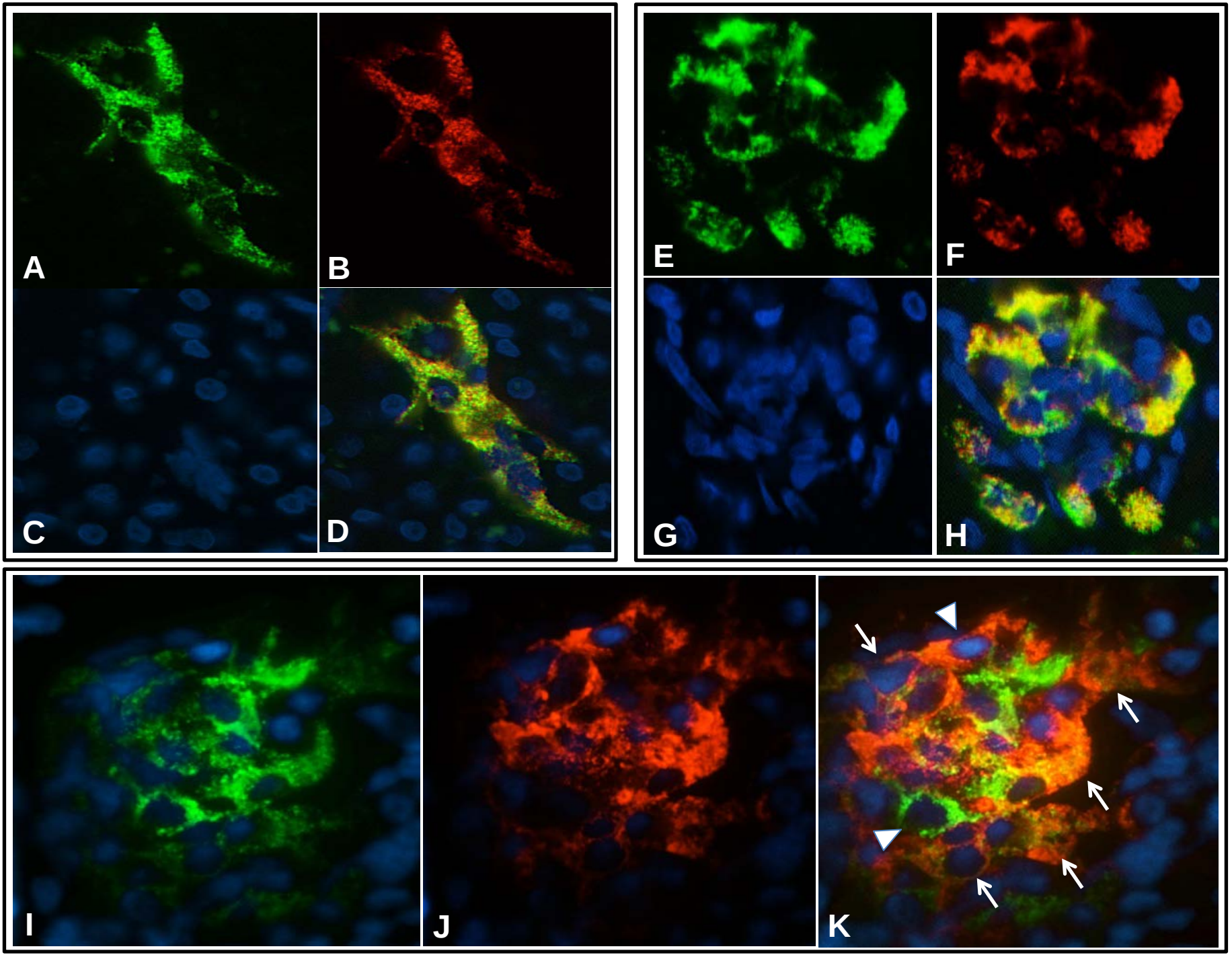
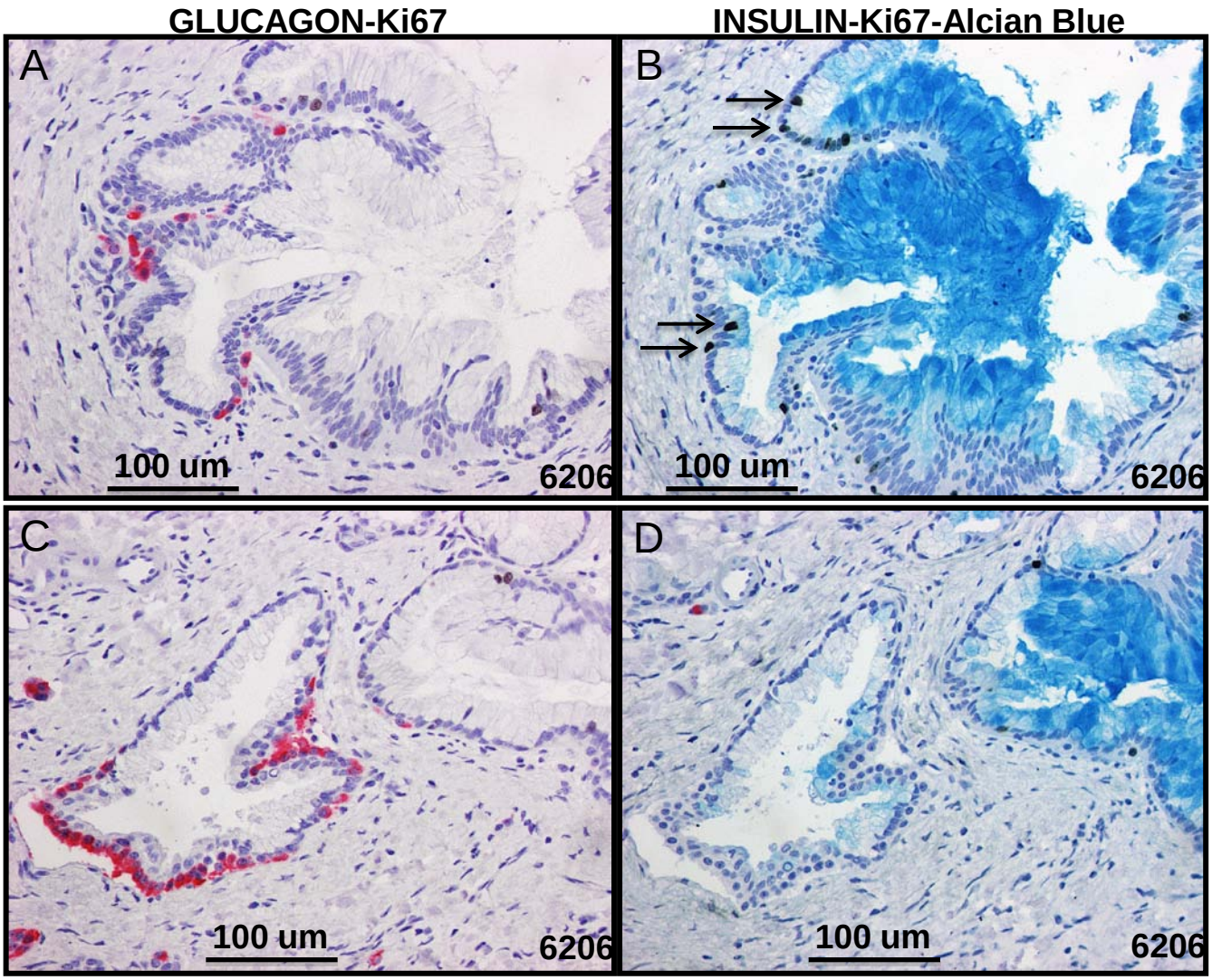
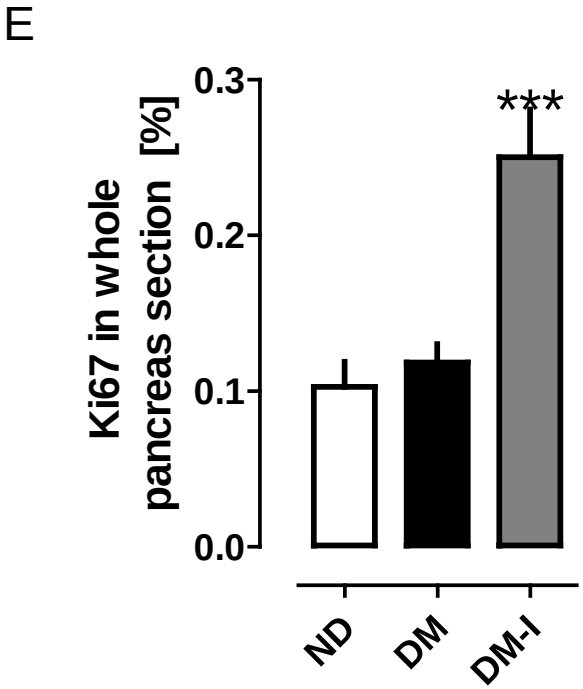

A
B
C
D
E
F
G
H
I
J
K

Figure 6. Insulin and glucagon co-expression.

Co-immunofluorescent images of islets from case 6185, Sitagliptin (A-H) showing endocrine cells co-expressing insulin (green) in A and E, glucagon (red) in B and F and merged in D and H. The proportion of endocrine cells per islet that were thus detected as co-expressing insulin and glucagon was markedly increased in type 2 diabetes following incretin therapy. I-K shows confocal images of an islet from case 6185 stained for (I) insulin (green), (J) glucagon (red) and (K) merged image showing a mixture of cells, some expressing insulin or glucagon only (arrow heads) and some showing co-expression (arrows). DAPI nuclear counterstain (blue).


GLUCAGON-Ki67
INSULIN-Ki67-Alcian Blue
A
B
C
D
100 um
6206


E
Ki67 in whole pancreas section [%]
0.3
0.2
0.1
0.0
***
ND
DM
DM-I

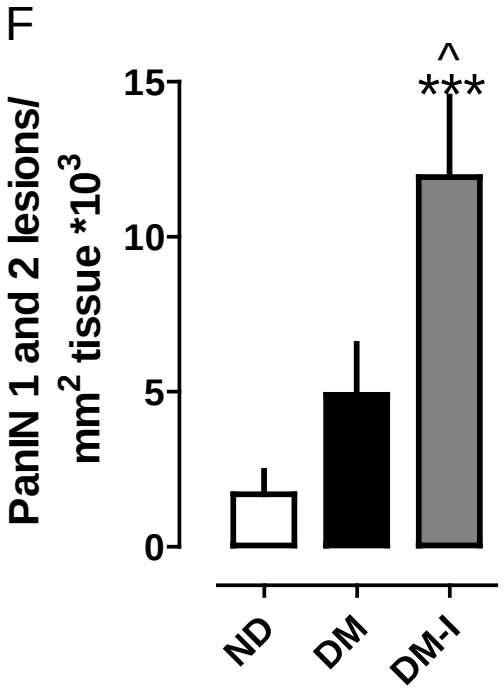

F
PanIN 1 and 2 lesions/ mm2 tissue *10^3
15
10
5
0
^
***
ND
DM
DM-I

Figure 7. Pancreatic intraepithelial neoplasia, endocrine complexes and cellular replication.

A-D photomicrographs showing sections with immunohistochemical staining for Ki67 (brown) and glucagon (pink) in PanIN lesions (A,C) with hematoxylin counterstain or Ki67 (brown) and insulin (pink) (B, D) with Alcian blue counterstain to highlight mucin. Glucagon-expressing endocrine cells are shown intimately associated with PanIN lesions to varying degrees. Foci of replication (arrows Ki67 nuclei) are also apparent (see supplementary figure 4 for additional examples of foci of increased replication in incretin treated pancreas). E. Pancreas cell replication is increased in type 2 diabetes treated by incretin therapy (Ki67). ***$p<0.0001$ DM-I vs DM and ND. F. Pancreatic intraepithelial neoplasia 1 and 2 (PanIN1 and 2) frequency (lesions per square mm $x10^3$ of pancreas) is increased in type 2 diabetes with incretin therapy. ^$p<0.01$ DM-I vs DM, ***$p<0.0001$ DM-I vs ND. *Abbreviations*. ND non diabetic controls, DM type 2 diabetes without incretin therapy, DM-I type 2 diabetes with incretin therapy.