Springer

Diabetologia

Evidence of increased islet cell proliferation in patients with recent onset type 1 diabetes

Journal:	*Diabetologia*
Manuscript ID:	Diab-10-0232.R2
Manuscript Type:	Article
Keywords:	1.01 Basic science, 2.10 Human, 3.02 Islets (all)

Evidence of increased islet cell proliferation in patients with recent onset type 1 diabetes

A. Willcox[1], S. J. Richardson[2], A. J. Bone[3], A. K. Foulis[4] and N. G. Morgan[1]

[1]Peninsula College of Medicine and Dentistry, University of Exeter, United Kingdom.

[2]Peninsula College of Medicine and Dentistry, University of Plymouth, United Kingdom.

[3]School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, United Kingdom.

[4]Department of Pathology, Glasgow Royal Infirmary, Glasgow, United Kingdom.

Corresponding Author:

Prof. Noel. G. Morgan
Institute of Biomedical and Clinical Science
Peninsula College of Medicine and Dentistry
Tamar Science Park
Derriford
Plymouth
PL6 8BU
Tel: +44 (0)1752 437412
Fax: +44 (0)1752 517846
E-mail: noel.morgan@pms.ac.uk

Word count

Abstract: 249

Main text: 3918

Key words: alpha cell, beta cell, human, immunohistochemistry, insulitis, Ki67, minichromosome maintenance protein 2, proliferation, type 1 diabetes.

Abbreviations: BB – biobreeding; ESM – electronic supplementary material; HIER – heat-induced epitope retrieval; ICI – insulin-containing islet; IDI – insulin-deficient islet; Mcm-2 – minichromosome maintenance protein 2.

Abstract

Aims/hypothesis In adults, the rate of beta-cell replication is normally very low but recent evidence implies that it may increase during insulitis. We have studied patients with recent-onset type 1 diabetes to establish whether islet cell proliferation is increased during the disease process.

Methods Paraffin-embedded pancreatic sections from 10 patients with recent onset type 1 diabetes, and a range of relevant controls, were stained by immunohistochemical techniques with antibodies against the proliferation markers, Ki67 and minichromosome maintenance protein-2 (Mcm-2). A combination staining technique involving both immunoperoxidase and immunofluorescence methods was developed to quantify the numbers of alpha- and beta-cells having Ki67-positive nuclei and to investigate the relationship between insulitis and islet cell proliferation.

Results In non-diabetic control subjects, only 1.1±0.3% of islets contained one or more $Ki67^+$ islet cells, whereas this proportion was increased markedly in recent-onset type 1 diabetes (10.88±2.5%; $p<0.005$). An equivalent increase in $Ki67^+$ staining occurred in both alpha and beta cells and was correlated positively with the presence of insulitis. A significant increase in the labelling of islet cells in type 1 diabetes was also seen when Mcm-2 staining was employed. Increased islet cell proliferation was not evident in 3 patients with longer duration type 1 diabetes or in 10 type 2 diabetic patients.

Conclusions/Interpretation This study reveals that both alpha and beta cells undergo a marked increase in proliferation during the progression of type 1 diabetes in humans. The results imply that islet cell proliferation is re-initiated in response to the autoimmune attack associated with type 1 diabetes.

Introduction

Type 1 diabetes is a T cell-mediated autoimmune disease in which the insulin-secreting beta cells of the islets of Langerhans are destroyed selectively [1]. Clinical symptoms arise when ~70% of the total beta cell mass is lost and this process follows a relatively protracted time course (months to years) during which the net rate of cell loss must exceed the rate of beta-cell replacement [2].

Beta-cell proliferation has been studied to only a limited extent in humans but the available evidence implies that this process occurs at relatively high levels during the first two years of life and declines rapidly thereafter [3, 4]. However, it is also clear that, at least in animals, beta cell proliferation can be re-induced in adulthood under conditions when insulin resistance is increased, for example during pregnancy or in obesity [5-7]. This suggests that beta cells may retain an intrinsic capacity to replicate but that they become largely quiescent during early childhood.

Recent evidence from a single case report [8] has revealed that enhanced proliferation of beta-cells occurred in an adult human subject who was characterised as "pre-diabetic" by virtue of the presence of circulating islet autoantibody positivity and active insulitis on histological examination of the pancreas. This proliferative response was observed prior to the onset of clinical symptoms, thereby raising the possibility that a compensatory increase in beta cell proliferation may occur early in the development of type 1 diabetes in humans. This would be consistent with evidence from animal models where enhanced beta-cell proliferation has also been seen early in the disease process [9-11], but this evidence differs from the conclusions of Butler et al. [12] who did not detect enhanced beta cell replication in pancreas sections recovered from a cohort of newly-diagnosed type 1 diabetes patients. It is important, therefore, to clarify whether enhanced beta cell proliferation occurs during the course of human type 1 diabetes. If this were to be the case, then a window of opportunity for therapeutic intervention might be considered as a means to replenish the beta cell mass, in parallel with attempts to limit the rate of autoimmune-mediated cell loss. One recent study has reported the detection of insulin-positive cells in 88% (37/42) of patients with long standing type 1 diabetes, therefore the window of opportunity in which intervention with potential regenerative therapies may be substantial [13].

In order to address this issue more fully, we have analysed pancreatic tissue from a cohort of patients with recent onset type 1 diabetes. Two independent markers of cell proliferation have been studied using both light and fluorescence microscopy to identify and quantify proliferating endocrine cells.

Methods.

Subjects 10 human pancreases recovered from patients with recent onset type 1 diabetes (disease duration ≤ 18 months) were selected from within a cohort used previously [14]. The selection was achieved randomly except that any specimens which had been post-fixed in mercuric chloride were excluded, as this fixative proved unsuitable for labelling with certain antibodies. The specimens used had been fixed in buffered *p*-formaldehyde, un-buffered formol saline or Bouin's fixative and they were all paraffin-embedded. 9 of the 10 pancreases were removed at autopsy (D1-D9) and one was recovered at the time of organ donation (D10). Nine of the patients were in the age range 1-23 years (mean 10.9±2.4). One female patient was 42, raising the overall mean to 14±3.8 years (Table 1). Pancreatic specimens from 14 non-diabetic (10 paediatric (C1-C10), 4 adult(C11-C14; surgical specimens, healthy regions utilised)), 10 type 2 diabetic patients (C15-C24), 3 patients with

long duration type 1 diabetes (C25-C27), 12 patients with pancreatitis (9 chronic (C28-C36), 2 autoimmune (C37-38) and 1 obstructive (C39)) and 9 non-diabetic patients which were age matched with the type 2 diabetic patients (C40-C48) were also studied. Full details are listed in Electronic Supplementary Material [ESM] Table 1. Sections of human tonsil and pancreatic lymph node were used as positive control tissues for markers of cell proliferation. The current investigation was performed with full ethical approval from the Research Ethics Committee, Glasgow Royal Infirmary, UK.

Immunohistochemistry Serial sections (4μm) were mounted on glass slides coated in (3-Aminopropyl)-triethoxysilane (Sigma Aldrich, Dorset, UK). Sections were processed and labelled using a standard immunoperoxidase technique for paraffin sections. Heat-induced epitope retrieval (HIER) was performed by heating the sections in a pressure cooker in a microwave oven on full power (800W) for 20 min followed by 20 min of cooling at room temperature. 1 mM EDTA (Sigma Aldrich) buffer pH 8.0 was used to unmask Ki67 and 10 mM citrate (Sigma Aldrich) buffer pH 6.0 was used for minichromosome maintenance protein 2 (Mcm-2). Details of all primary antibodies are listed in Table 2. Sections were incubated in primary antibody for 1 h at room temperature. Dako REAL™ Envision™ Detection System was used for antigen detection. Sections were counterstained with hematoxylin (Dako). Some slides were processed in the absence of primary antibody or with isotype control antisera to confirm the specificity of labelling.

Combined immunoperoxidase and immunofluorescence immunohistochemistry A combination technique was used in which sections were dual stained using both the immunoperoxidase technique described above, followed by immunofluorescence labelling of the same sections. Anti-Ki67 and anti-Mcm-2 were detected on separate sections using the immunoperoxidase technique while the endocrine cells and lymphocytes were detected using immunofluorescence. After counterstaining with hematoxylin, Ki67 immunoperoxidase-stained sections from patients with recent on set type 1 diabetes and the non-diabetic controls were incubated with primary antibodies against either insulin and glucagon or insulin and somatostatin or insulin and CD45 or glucagon and CD45. Mcm-2 stained sections were further incubated with antibodies against either insulin or CD45. Sections from chronic pancreatitis patients were further incubated in a cocktail of antibodies against insulin and glucagon. The antibodies were detected with goat AlexaFluor® 488 or 568 conjugated secondary antibodies (Invitrogen, Paisley, UK). DAPI (1:1000, Invitrogen) was included in the final secondary incubation to stain cell nuclei. Nine of the 10 type 1 diabetic patients were found to be suitable for analysis by this method, while the tissue from one patient failed to absorb the DAPI nuclear stain. Sections were mounted in Vectashield hard-set mounting medium (Vector Laboratories, Peterborough, UK) under glass cover slips.

The sections were analysed initially using the brightfield function of a Nikon Eclipse 80*i* microscope (Nikon, Surrey, UK) and a monochrome photomicrograph was captured for each islet that contained at least one Ki67+ cell. The fluorescence imaging function of the microscope was then used to capture immunofluorescence staining. The images were precisely overlaid using NIS-Elements BR 3.0 software (Nikon) to determine the endocrine cell sub-type of the Ki67+ or Mcm-2+ cells.

Staining analysis Islets were identified morphologically in each pancreas section after counter-staining with hematoxylin. Initially all islets on each section from every patient were assessed for the presence of Ki67+ or Mcm-2+ nuclei and the percentage of islets containing one or more immunopositive cells per section was recorded. Using the combination imaging technique described above, the identity of each of the cells having Ki67+ nuclei was

determined. In islets which were infiltrated with immune cells (which could be readily identified by their unique morphology) any Ki67+ cell that was evidently an immune cell was excluded from the analysis. A Ki67 labelling index was determined for both the alpha and beta cells in the non-diabetic control and type 1 diabetic patient groups according to the percentage of each islet cell type that was positively stained for Ki67. Cell counting was carried out using Adobe® Photoshop® CS4 software. Islets were categorised as insulitis-positive if there were ≥5 CD45+ cells within the islet perimeter [14].

Statistical analysis The percentage of Ki67+ or Mcm-2+ islets per section is expressed as the mean ± SEM and statistical significance was calculated by Students t-test using SPSS 16 software. The statistical significance of differences in the labelling index of islet cells and also the insulitis correlation was calculated using χ^2 analysis.

Results

The percentage of islets with Ki67 positive islet cells is increased in recent onset type 1 diabetic patients compared to non diabetic controls. For the initial analysis, one pancreas section from each of 10 recent onset type 1 diabetic patients and 14 age-matched non-diabetic controls was stained with an anti-Ki67 antibody and the percentage of islet sections having at least 1 Ki67+ nucleus was calculated. A mean of only 1.1±0.3% Ki67+ islets per pancreas section (range: 0-1.5%) was recorded in the non-diabetic controls. This number was increased by more than 10 fold in the recent onset type 1 diabetic group to 10.9±2.5% ($p<0.005$) of islets per section (range: 1.5-31.8%) (Fig. 1a; Table 3). A typical Ki67+ islet from a type 1 diabetic patient is shown in Fig. 1b.

Overall, a total of 1280 islet sections were examined in the type 1 diabetic group and 4031 islet sections in the controls. Within the type 1 diabetic group, a mean of 37.1±8.2% (range: 6.7-78.4%) of the total islet sections were insulin-positive. Of the population of islet sections that were Ki67+, 23% were insulin-negative, suggesting that the increase in proliferation was not restricted solely to beta cells. The mean area per islet was calculated for the recent-onset type 1 patients and the non-diabetic controls. These values were 18227±1054 μm^2 for insulin positive and 9017±1139 μm^2 for insulin-negative islets in the type 1 diabetic group and 12265±1095 μm^2 in the non-diabetic controls.

The increase in Ki67+ islet cells seen in recent on-set type 1 diabetes occurs in both alpha and beta cells. To determine which islet cells expressed Ki67 in recent-onset type 1 diabetes, sections from 9 patients were analysed in greater detail. Each section was co-stained with anti-Ki67 (immunoperoxidase) and combinations of anti-insulin, anti-glucagon and anti-somatostatin (immunofluorescence). The sections were photomicrographed under brightfield illumination to capture images of the islets containing at least one Ki67+ islet cell. Fluorescence images of the staining patterns of insulin, glucagon and somatostatin were captured subsequently and the images were overlaid (Fig. 2a-g; [ESM 2 and 3]).

The Ki67 labelling index was 0.006% for alpha cells and 0.008% for beta cells in the sections from control subjects. The labelling index of both cell types increased significantly in the recent onset type 1 diabetic group to 0.066% (alpha) and 0.07% (beta cells) respectively (χ^2 $p<0.001$) (Fig. 3). The number of Ki67+ delta cells was insufficient to allow accurate quantification of a labelling index.

The topography of the staining patterns precluded unequivocal identification of 10-20% of the Ki67+ islet cells in both the recent onset type 1 diabetic pancreatic sections and the non-diabetic controls.

Increased proliferation of islet *cells in type 1 diabetes is also seen using a second marker of cell proliferation.* Ki67 is a widely used marker of cell proliferation however, it was considered important to confirm the presence of proliferating cells with a second proliferation-specific protein, unrelated to Ki67. Mcm-2 was chosen for this purpose and pancreas sections from the 10 type 1 diabetic and 14 non-diabetic control patients were stained with an anti-Mcm-2 antibody using the immunoperoxidase technique. A mean of 2.4±0.5% (range: 0-7.1%) islets per section had at least one Mcm-2+ cell in the control group. This was a statistically higher percentage than that found with the Ki67 antibody in the equivalent subjects ($p<0.05$) which may reflect the longer period of expression of Mcm-2 vs Ki67 during the cell cycle, as revealed by the staining patterns seen with these markers in tonsil epithelium [ESM 4]. In this tissue, the oral mucosa cells divide at the base of the stratified squamous epithelium and migrate towards the surface after division. Mcm-2+ cells were detected further from the dividing layer than those stained with Ki67, consistent with the longer persistence of Mcm-2 after mitosis. Staining of sections from the type 1 diabetic group confirmed a marked increase in the rate of proliferation of islet cells (to 11±2.6% of islets; $p<0.001$ vs control; Fig. 4a). A representative islet section stained positively for Mcm-2 is shown in Fig. 4b.

Pancreas sections from 3 of the 10 recent onset type 1 diabetic patients were double-stained with anti-Mcm-2 and anti-insulin in order to assess the percentage of Mcm-2+ beta cells. 54 Mcm-2+ islets were examined and, among these, a total of 142 Mcm-2+ cells were detected. Of the Mcm-2+ cells, 60 (42%) were dual-positive for insulin, thereby confirming that these were beta cells (Fig. 4c and d).

To confirm that the majority of Mcm-2+ cells were islet cells rather than infiltrating lymphocytes, dual staining was performed with anti-Mcm-2 and anti-CD45. 21 Mcm-2+ islets that were heavily infiltrated with CD45+ lymphocytes were examined in detail. Very few of the infiltrating CD45+ cells were co-positive for Mcm-2 (Fig. 4e). Peripancreatic lymph nodes were often present adjacent to the pancreas sections and dual staining revealed the presence of Mcm-2+ CD45+ cells, demonstrating that immunoperoxidase staining of the nucleus with anti-Mcm-2 had not interfered with the ability of the CD45 antibody to stain the lymphocyte cell surface (Fig. 4f).

The presence of Ki67+ islet cells correlates with insulitis in type 1 diabetes. Pancreas sections were stained to assess the relationship between insulitis and Ki67-positivity in islet cells. Insulitis was defined as the presence of 5 or more CD45+ lymphocytes within the islet perimeter [14]. A total of 1091 islet sections were examined from 8 patients and 247 of these (22.6%) were insulitis-positive. Among this group, 44/247 islets (17.8%) contained one or more Ki67+ islet cell. Of these 44 islets, 42 were also insulin-containing islets. By contrast, only 22 of the 844 insulitis negative islets (2.6%) contained a Ki67+ islet cell. Of these 22 islets, 7 were insulin-containing (Fig. 5a and b). Thus, there was a 7-fold increase in the frequency with which Ki67+ islet cells were detected in islets containing infiltrating immune cells compared to those that were insulitis-negative (χ^2 $p<0.001$). In order to verify this conclusion, we also compared the proportion of insulin-containing islets that were both Ki67+ and inflamed (42 of 405 (10.4%)) with those that were Ki67+ but not inflamed (7 of 405 (1.7%)). This large difference is consistent with the proposition that proliferation correlates with the inflammatory response. Additional support was obtained by a direct

comparison of the Ki67 labelling index for Ki67+/Insulin+ cells in inflamed vs non-inflamed islets, which yielded significantly different values of 0.097% and 0.004% (P<0.0005), respectively (Fig. 5c).

Increased Ki67+ labelling of islet cells is not maintained in patients having longer duration type 1 diabetes. To establish whether the increase in proliferating islet cells is persistent, pancreatic sections from 3 type 1 diabetic patients with a longer disease duration (6, 12 and 12 years respectively) were stained with the anti-Ki67 antibody. One of these patients (disease duration of 12 years) still retained some residual beta cells within 53/79 of their islet sections while the other patients were entirely beta cell deficient in the available sections. No Ki67+ islet cells were detected in any of the 561 islet sections examined in these patients (Table 3).

Increased Ki67+ labelling of islet cells is not observed in patients with type 2 diabetes. To determine whether the increase in islet cell proliferation was restricted to patients with (recent-onset) type 1 diabetes, pancreatic sections from 10 patients with type 2 diabetes were also stained with the Ki67 antibody. A total of 1436 islets were examined and the mean percentage of Ki67+ islets per section (1.5±0.16%; 3 patients had no Ki67+ cells) was statistically lower than that of the recent onset type 1 diabetic group (P<0.005) but not different from the non-diabetic control patients (P=0.55; Table 3). The type 2 diabetic patients were also compared with 9 age matched non-diabetic control patients stained for Ki67. In this group a mean of 0.97±0.37% of islets per section contained Ki67+ cells which was not statistically different from the type 2 diabetic patients (P=0.5; Table 3).

Increased Ki67+ labelling of islet cells is not observed in patients with pancreatitis. Since the increased proliferation detected in patients with recent-onset type 1 diabetes correlated with immune cell infiltration, it was also considered important to examine the pancreas of patients with a different inflammatory disease of the pancreas, pancreatitis. Accordingly, pancreatic sections from 12 patients with either chronic, autoimmune or obstructive pancreatitis were stained using the combination technique for Ki67, insulin and glucagon. Of the 12 samples analysed (2989 islets in total), three did not contain any Ki67+ cells within islets. In the remaining nine sections 82 out of 2651 islets examined contained a total of 139 Ki67+ cells. Of these, 42 were identified as endocrine cells (14 alpha and 26 beta) and 99 were non-endocrine cells (Fig. 6a-c). In these samples no attempt was made to study small clusters of endocrine cells associated with pancreatic ducts.

Discussion

The rate of islet endocrine cell proliferation in humans varies with age. A wave of proliferation occurs early in postnatal life as the endocrine pancreas is released from the intrauterine environment and is remodelled to meet the demands of independent living and changing nutrition [3]. This period is, however, short-lived, and is followed by a rapid decline in the proliferation rate which then stabilises at an extremely low level that is maintained throughout childhood and into adult life. A similar situation may also pertain in other mammalian species although, in some species, islet cell proliferation can be re-induced during adulthood by the imposition of increased metabolic demand (e.g. during obesity) and also in response to the hormonal adaptations associated with pregnancy [6, 7]. Importantly, we now demonstrate that islet cell proliferation is increased in humans during the ongoing process of insulitis and autoimmunity associated with the recent onset of type 1 diabetes.

We have employed immunohistochemical detection of two independent proteins expressed uniquely during cell division, to study the proliferation of islet cells. Ki67 is widely used as a

marker of cell division in fixed tissue as it is expressed specifically during all stages of the cell cycle but is degraded before cells re-enter G0 [15]. Mcm-2 plays a role in DNA replication and is expressed from early G1 through the remaining phases of the cell cycle but, is degraded prior to entry into G0 [16]. We analysed both proteins and found that they were detected at higher frequency in the nuclei of islet cells from patients with recent onset type 1 diabetes than in non-diabetic controls. Detailed analysis revealed a 10-fold increase in the proportion of islets displaying one or more Ki67+ cells in the patient samples vs controls. The increase was somewhat lower when the labelling of Mcm-2 was assessed (approximately 5-fold) but remained statistically significant. The reduction in the magnitude of the response seen when assessing Mcm-2 was principally due to the larger proportion of islet sections from control subjects which displayed Mcm-2 immunopositivity compared to those which were positive for Ki67. The reasons for this difference may reflect the varying periods of the cell cycle when each protein is expressed. Analysis of the islet cells which display increased proliferation in the islets of patients with recent onset type 1 diabetes revealed that the labelling index of both alpha and beta cells for Ki67+ was enhanced by approximately 10-fold. Thus, unlike the process of immune-mediated islet cell destruction (which is entirely beta cell specific) the proliferative response appears not to display such cell specificity.

The increase in islet cell proliferation correlated closely with the presence of insulitis suggesting that a signal(s) emanating from infiltrating immune cells might mediate the enhanced proliferative response. Consistent with this hypothesis, an increase in islet cell proliferation has also been reported in the inflamed islets of a patient classified as having 'pre-diabetes' [8]. In addition, an 89 year old man recently diagnosed with type 1 diabetes, had increased Ki67+ islet cells and it was noted that this patient also had insulitis in a large percentage of islets [17]. Taken together, these results support the suggestion that human islet cell proliferation can be increased during islet inflammation. Moreover, our finding that patients with a long duration of diabetes do not display islet cell proliferation also support this conclusion since islet inflammation was minimal in these patients.

Additional support for the possible involvement of immune-derived signals as mediators of islet cell proliferation comes from the NOD mouse and the biobreeding (BB) rat. In the NOD model, beta cell proliferation is increased during the pre-diabetic phase and this has been attributed to the presence of insulitis in the relevant islets [9, 10]. The BB rat also has a higher than normal islet cell Ki67 labelling index during the pre-diabetic period when the islets are infiltrated by mononuclear cells [18]. Similar findings have also been reported in a partial pancreatectomy model in the LEW.Han rat [19] although, unlike our present findings in man, the alpha cell proliferation was unchanged in these animals.

Examination of pancreatic sections from patients with pancreatitis, another inflammatory disease of the pancreas, revealed low numbers of Ki67+ alpha or beta cells which was not consistent with a large-scale proliferative response. However, a significant increase in beta cell replication has been reported previously in sections from patients with severe pancreatitis suggesting that, under these circumstances, the immune cells may elaborate a stimulus for endocrine cell proliferation [20]. One study has also reported neogenesis of duct epithelial cells in to insulin-producing cells in patients with chronic pancreatitis [21].

We have not characterised the nature of the putative immune cell derived-signal which may promote islet cell proliferation but very low concentrations of certain pro-inflammatory cytokines (including IL-1β) can elicit beta cell proliferation in isolated human islet preparations [22]. Higher doses of these same cytokines are cytotoxic suggesting that the gradient of concentration to which islet cells are exposed may determine their ultimate fate.

Conceivably, therefore, the initial influx of immune cells might promote endocrine cell proliferation and this response is ultimately over-ridden by a net cytotoxicity as the concentration of immune mediators increases further. It is important to note, however, that we did not detect an increase in Ki67+ islet cells in pancreas sections from patients with type 2 diabetes. This form of diabetes has recently been shown to be associated with a low-level macrophage infiltration of the islets but there is no lymphocyte infiltration [14, 23, 24]. Hence, this implies that a signal other than IL-1β (which is secreted by macrophages but not by T- or B-lymphocytes) may be primarily responsible for mediating the proliferative response. The finding that islet cell proliferation is not increased in patients with type 2 diabetes also implies that hyperglycaemia is unlikely to represent a primary stimulus for the proliferative response since this is a feature of both forms of the disease.

The present results differ from the recent findings of Butler et al. who did not find any increase in beta cell proliferation in a cohort of patients with recent onset type 1 diabetes [12]. If, as we propose, the proliferative response reflects an early response to insulitis, then this may have been undetectable in several of the samples examined by Butler et al. [12] since four of the nine patients studied did not display insulitis. However, other undefined factors might also account for the differences.

In summary, the current study reveals that islet cell proliferation is increased in the islets of patients with recent onset type 1 diabetes. This correlates with inflammation and suggests that the immune cells elaborate a signal which promotes islet cell mitosis. These results suggest that, if mechanisms could be found to facilitate cell proliferation during the pre-diabetic phase, then it may be possible to facilitate the regeneration of beta cells as a therapeutic intervention in patients progressing to type 1 diabetes.

Acknowledgments

We are grateful to the Juvenile Diabetes Research Foundation for funding this study.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

References

[1] Foulis AK (1996) The pathology of the endocrine pancreas in type 1 (insulin-dependent) diabetes mellitus. Apmis 104: 161-167
[2] Atkinson MA, Gianani R (2009) The pancreas in human type 1 diabetes: providing new answers to age-old questions. Current opinion in endocrinology, diabetes, and obesity 16: 279-285
[3] Meier JJ, Butler AE, Saisho Y, et al. (2008) Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 57: 1584-1594
[4] Cnop M, Hughes SJ, Igoillo-Esteve M, et al. (2009) The long lifespan and low turnover of human islet beta cells estimated by mathematical modelling of lipofuscin accumulation. Diabetologia 53: 321-330
[5] Dhawan S, Georgia S, Bhushan A (2007) Formation and regeneration of the endocrine pancreas. Current opinion in cell biology 19: 634-645
[6] Sorenson RL, Brelje TC (1997) Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones. Hormone

and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme 29: 301-307
[7] Rieck S, Kaestner KH (2009) Expansion of beta-cell mass in response to pregnancy. Trends in endocrinology and metabolism: TEM 21: 151-158
[8] In't Veld P, Lievens D, De Grijse J, et al. (2007) Screening for insulitis in adult autoantibody-positive organ donors. Diabetes 56: 2400-2404
[9] Sreenan S, Pick AJ, Levisetti M, Baldwin AC, Pugh W, Polonsky KS (1999) Increased beta-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse. Diabetes 48: 989-996
[10] Sherry NA, Kushner JA, Glandt M, Kitamura T, Brillantes AM, Herold KC (2006) Effects of autoimmunity and immune therapy on beta-cell turnover in type 1 diabetes. Diabetes 55: 3238-3245
[11] Nir T, Melton DA, Dor Y (2007) Recovery from diabetes in mice by beta cell regeneration. The Journal of clinical investigation 117: 2553-2561
[12] Butler AE, Galasso R, Meier JJ, Basu R, Rizza RA, Butler PC (2007) Modestly increased beta cell apoptosis but no increased beta cell replication in recent-onset type 1 diabetic patients who died of diabetic ketoacidosis. Diabetologia 50: 2323-2331
[13] Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC (2005) Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia 48: 2221-2228
[14] Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG (2009) Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol 155: 173-181
[15] Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H (1984) Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133: 1710-1715
[16] Todorov IT, Attaran A, Kearsey SE (1995) BM28, a human member of the MCM2-3-5 family, is displaced from chromatin during DNA replication. The Journal of cell biology 129: 1433-1445
[17] Meier JJ, Lin JC, Butler AE, Galasso R, Martinez DS, Butler PC (2006) Direct evidence of attempted beta cell regeneration in an 89-year-old patient with recent-onset type 1 diabetes. Diabetologia 49: 1838-1844
[18] Bone AJ, Walker R, Dean BM, Baird JD, Cooke A (1987) Pre-diabetes in the spontaneously diabetic BB/E rat: pancreatic infiltration and islet cell proliferation. Acta endocrinologica 115: 447-454
[19] Lampeter EF, Tubes M, Klemens C, et al. (1995) Insulitis and islet-cell antibody formation in rats with experimentally reduced beta-cell mass. Diabetologia 38: 1397-1404
[20] Campbell-Thompson M, Dixon LR, Wasserfall C, et al. (2009) Pancreatic adenocarcinoma patients with localised chronic severe pancreatitis show an increased number of single beta cells, without alterations in fractional insulin area. Diabetologia 52: 262-270
[21] Phillips JM, O'Reilly L, Bland C, Foulis AK, Cooke A (2007) Patients with chronic pancreatitis have islet progenitor cells in their ducts, but reversal of overt diabetes in NOD mice by anti-CD3 shows no evidence for islet regeneration. Diabetes 56: 634-640
[22] Maedler K, Schumann DM, Sauter N, et al. (2006) Low concentration of interleukin-1beta induces FLICE-inhibitory protein-mediated beta-cell proliferation in human pancreatic islets. Diabetes 55: 2713-2722
[23] Ehses JA, Perren A, Eppler E, et al. (2007) Increased number of islet-associated macrophages in type 2 diabetes. Diabetes 56: 2356-2370
[24] Richardson SJ, Willcox A, Bone AJ, Foulis AK, Morgan NG (2009) Islet-associated macrophages in type 2 diabetes. Diabetologia 52: 1686-1688

Table 1 Details of patients with recent onset type 1 diabetes

Patient	Age	Gender	Duration of diabetes	Cause of death	No. Islets	No. Ki67+ Islets	No. ICIs	No. ICI with Insulitis	No. IDI with Insulitis
D1	1	F	3 days	Ketoacidosis	149	11	10	10	21
D2	4	F	3 weeks	Ketoacidosis	132	16	32	22	5
D3	6	F	<1 week	Ketoacidosis	37	4	29	10	0
D4	7	F	Unspecified but recent	Ketoacidosis	90	8	42	3	2
D5	11	F	5 days	Ketoacidosis	132	14	30	26	15
D6	13	M	2 days	Ketoacidosis	86	7	21	14	2
D7	15	M	6 months	Ketoacidosis	204	3	21	9	8
D8	18	M	4 months	Hypoglycaemia	73	23	57	17	0
D9	23	M	2 weeks	Ketoacidosis	103	12	65	6	0
D10	42	F	18 months	Glioma raised intracranial pressure	274	17	45	24	7

Table 2. Details of primary antibodies used.

Antigen	Cell type	Species	Dilution	Source
Ki67	Proliferating	Mouse	1/200	Dako, Ely, Cambridgeshire, UK
Mcm-2	Proliferating	Rabbit	1/2000	Abcam, Cambridge, UK
Insulin	Beta	Guinea pig	1/600	Dako
Glucagon	Alpha	Rabbit	1/300	Dako
Somatostatin	Delta	Rabbit	1/200	Abcam
CD45	Lymphocytes	Mouse	1/750	Dako

Table 3 The mean percentage of Ki67+ islets detected in the patient groups.

Patient group	No. patients	No. islets examined	Mean(±SEM) % Ki67+ islets
Non-diabetic controls	14	4031	1.1±0.3
Recent onset type 1 diabetics	10	1280	10.9±2.5[a,b]
Long duration type 1 diabetics	3	561	0
Type 2 diabetics	10	1436	1.5±1.6[c]
Non-diabetic controls age matched with type 2 diabetics	9	3078	0.97±0.37[d]

[a]significantly greater than non-diabetic controls ($p<0.005$);

[b]significantly greater than patients with type 2 diabetes ($p<0.005$);

[c]not significantly different from non-diabetic controls ($p=0.55$).

[d]not significantly different from type 2 diabetic patients ($p=0.5$)

Figure Legends

Fig. 1 Ki67+ islet cell staining. (**a**) The histogram shows the mean percentage of islets per section with ≥1 Ki67+ cells in the recent on set type 1 diabetic and non-diabetic control patients (** P < 0.005). (**b**) A photomicrograph of Ki67+ cells (brown) in an islet of a type 1 diabetic patient, counterstaining with hematoxylin is seen in purple. Scale bar = 20 µm.

Fig. 2 Photomicrographs of islets from type 1 diabetic patients stained using the combination technique to determine the cell type of the Ki67+ nuclei. (**a**) An islet showing insulin (green), glucagon (red) and Ki67 (dense black nuclei). The white and orange boxes highlight the areas which are focused on in images (**b**) and (**c**) respectively which demonstrate Ki67+, insulin+ cells indicative of proliferating beta cells. (**d**) An islet showing insulin (green), glucagon (red) and Ki67 (dense black nuclei). The white box highlights the area focused on in image (**e**) which indicates a Ki67+ alpha cell. In images **a**-**e** cell nuclei are stained with DAPI (blue). (**f**) A brightfield image of the islet demonstrated in (**a**). Ki67+ nuclei are visible in brown and counterstaining with hematoxylin in purple. The black boxes highlight the two areas which are focused upon in images (**b**) and (**c**). (**g**) A brightfield image of the islet demonstrated in (**d**). The black box highlights the nucleus that is focused upon in image (**e**). Scale bars = 20 µm.

Fig. 3 Ki67 labelling index for alpha and beta cells. The red bars show the percentage of total Ki67+ alpha cells detected in both the non-diabetic control group and the recent onset type 1 diabetic group (***P<0.001) and the green bars indicate the percentage of Ki67+ beta cells detected in both patient groups (***P<0.001).

Fig. 4 Mcm-2+ islet cell staining. (**a**) The histogram shows the mean percentage of islets per section with ≥1 Mcm-2+ cells in type 1 diabetic and non-diabetic control patients (* P < 0.05). (**b**) Photomicrograph demonstrating the presence of Mcm-2+ cells within an islet of a type 1 diabetic patient. (**c**) Combination staining of Mcm-2 (dense black nuclei) and insulin (green) indicated the presence of proliferating beta cells in a type 1 diabetic patient. The white box highlights the area which is focused upon in (**d**) showing a Mcm2+ insulin+ cell. (**e**) The combination technique was used to stain the type 1 diabetic patients for Mcm-2 (dense black nuclei indicated by red arrows) and CD45 (green) to demonstrate that the increased number of Mcm-2+ nuclei in the type 1 diabetic patients vs. controls is not due to lymphocyte staining. (**f**) Staining of a pancreatic lymph node (on the same section as image **e**) demonstrates that the Mcm-2 immunoperoxidase staining does not disrupt the cells ability to also stain positively for CD45. Scale bars = 20 µm.

Fig. 5 Ki67+ islets show a correlation with insulitis in type 1 diabetic patients. (**a**) The flow diagram shows the distribution of Ki67+ islets in 8 type 1 diabetic pancreas sections. The total number of islets (1091) was divided into those which were insulitis+ or insulitis-. The number of Ki67+ islets in each category was counted and 66 Ki67+ islets were present in total. The Ki67+ islets were subdivided into those which were insulin+ or insulin- according to the presence or absence of positive insulin immunostaining. (**b**) The photomicrograph demonstrates the presence of insulitis in an islet stained for insulin (green), CD45 (red),

DAPI for nuclei in blue and Ki67 (dense black nuclei highlighted by the white arrow) using the combination technique, scale bar = 20 µm. (**c**) The histogram represents the percentage of Ki67+ insulin+ cells in insulitis-negative and insulitis-positive islets in recent onset type 1 diabetic patients. The insulitis-negative group comprised a total of 844 islets and the insulitis-positive group contained 247 islets, (***P<0.0005).

Fig. 6 A Ki67+ islet in a patient with chronic pancreatitis. (**a**) The photomicrograph shows insulin (green), glucagon (red) and Ki67 (dense black nuclei) and DAPI (blue). (**b**) The image shows the region from image A which is highlighted by the white box at the bottom left. The green insulin staining is indicative of the black nucleus having a beta cell phenotype. (**c**) The image shows the region from image **(a)** which is highlighted by the top right white box. The red glucagon staining surrounding the black nuclei indicates that this nucleus has an alpha cell phenotype. Scale bar = 20 µm.

Figure 1.

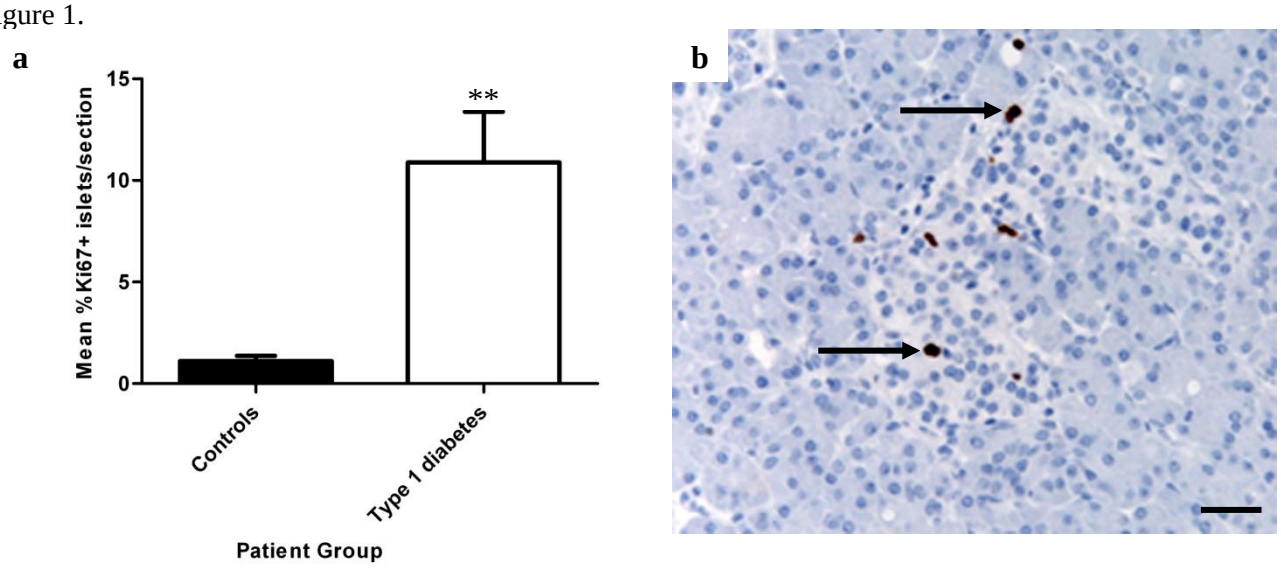


Figure 2.

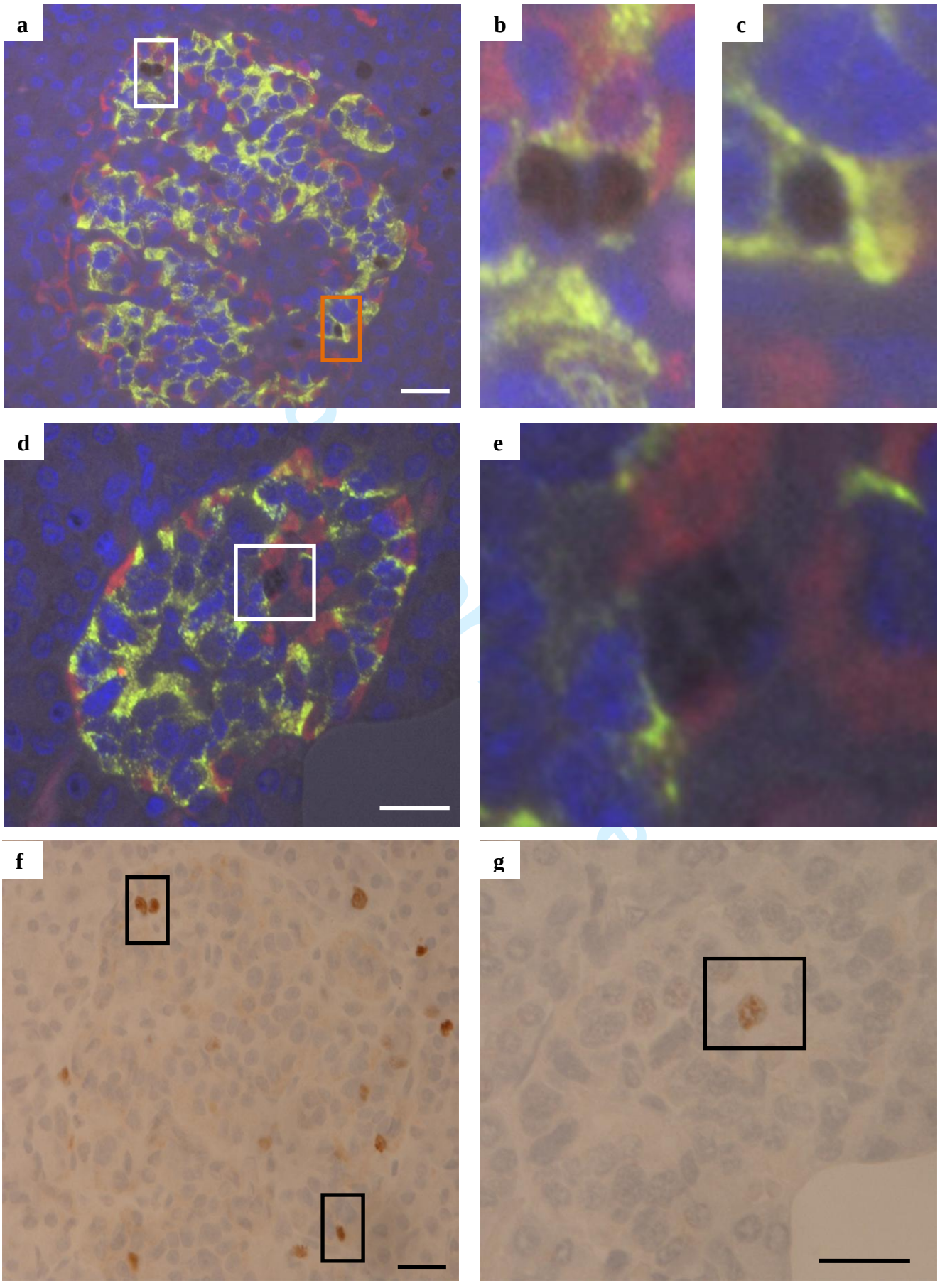


Figure 3.

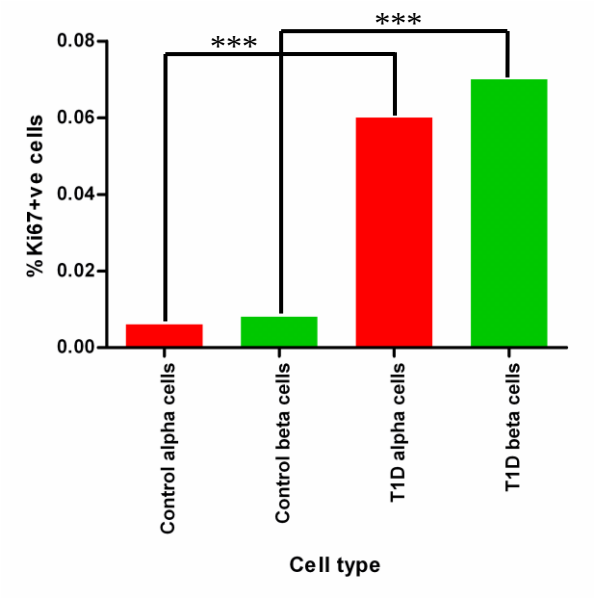


Figure 4.

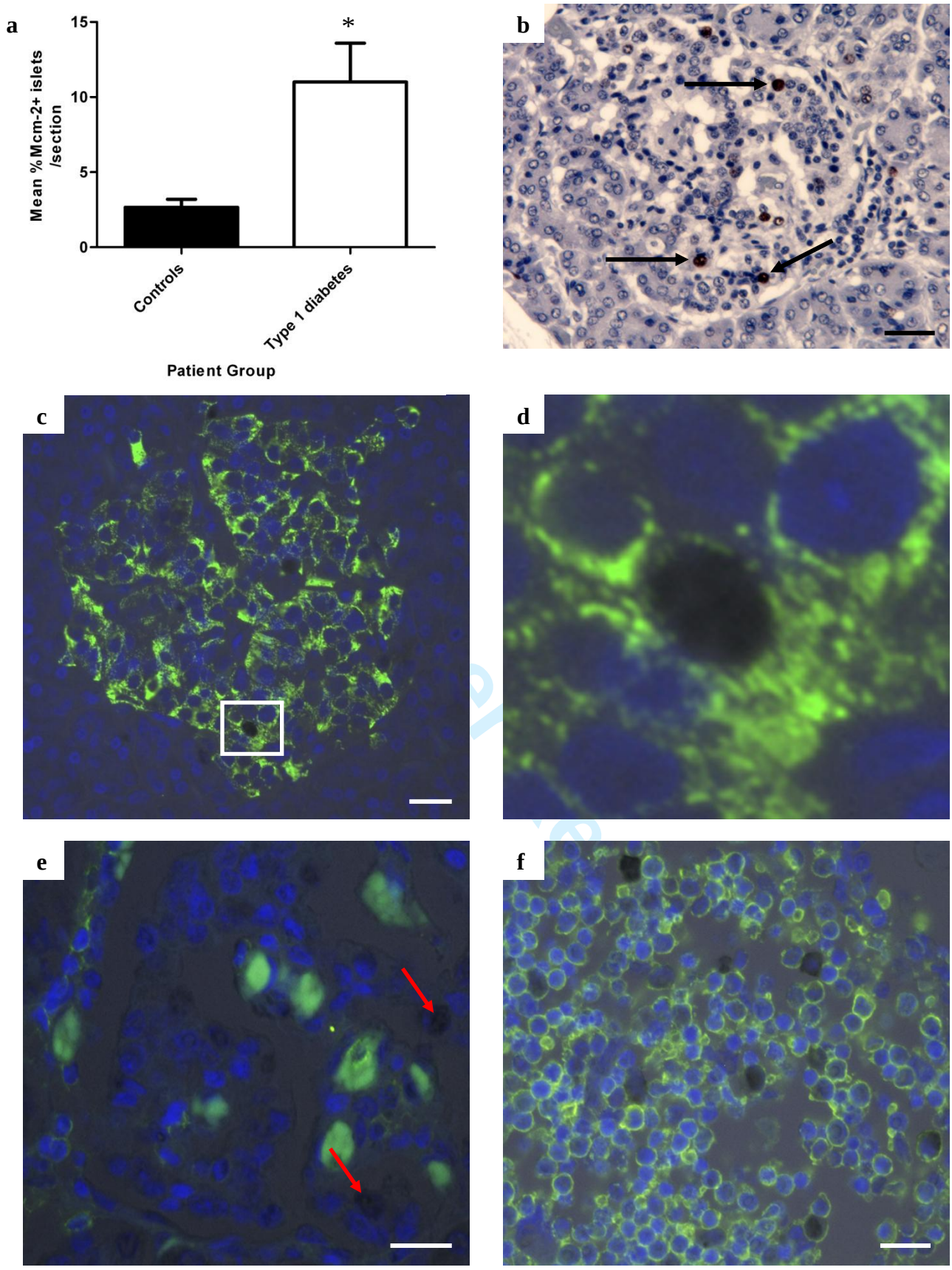

a
15
10
5
0
*
Mean % Mcm-2+ islets /section
Controls
Type 1 diabetes
Patient Group
b
c
d
e
f

Figure 5.

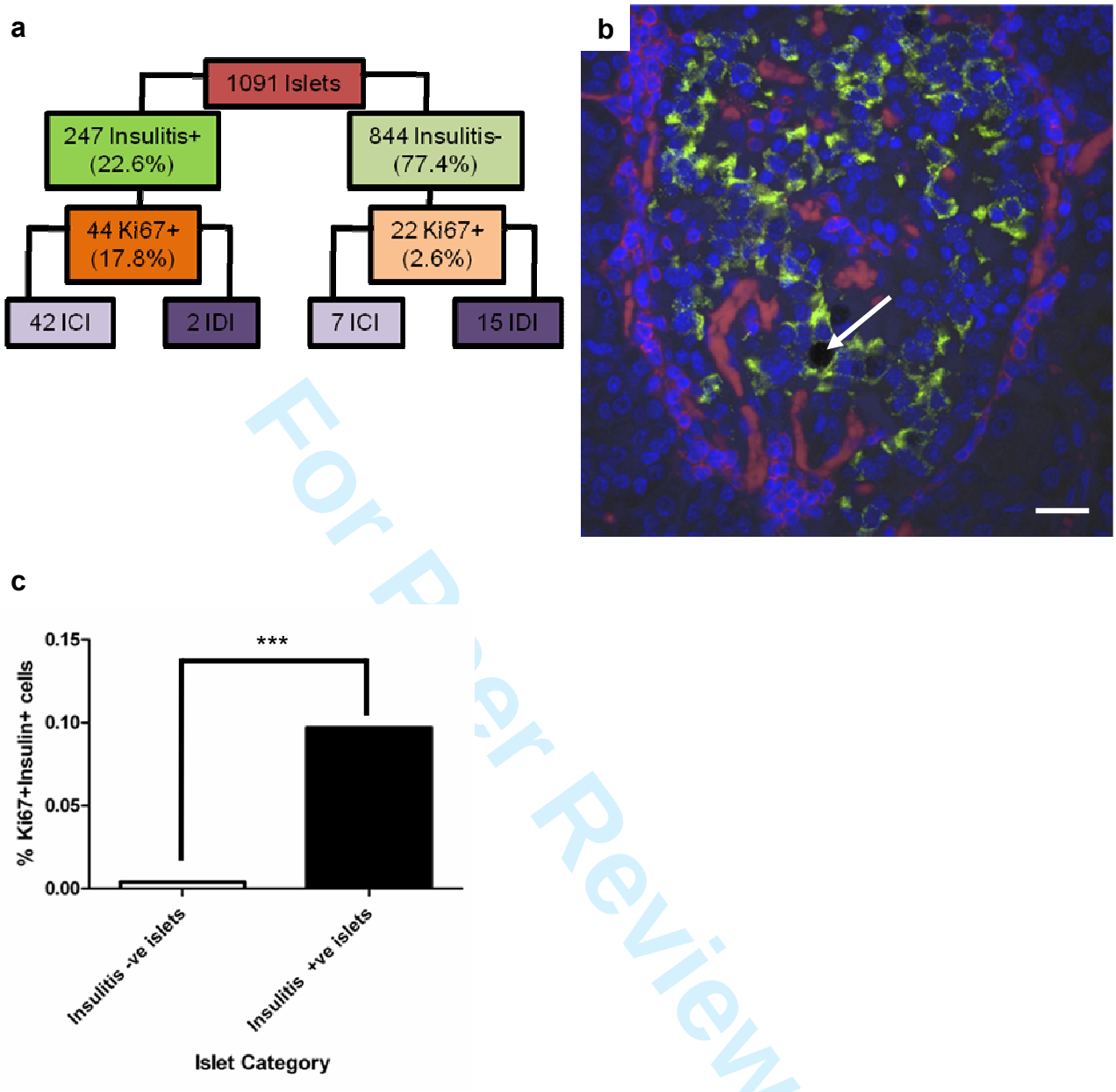
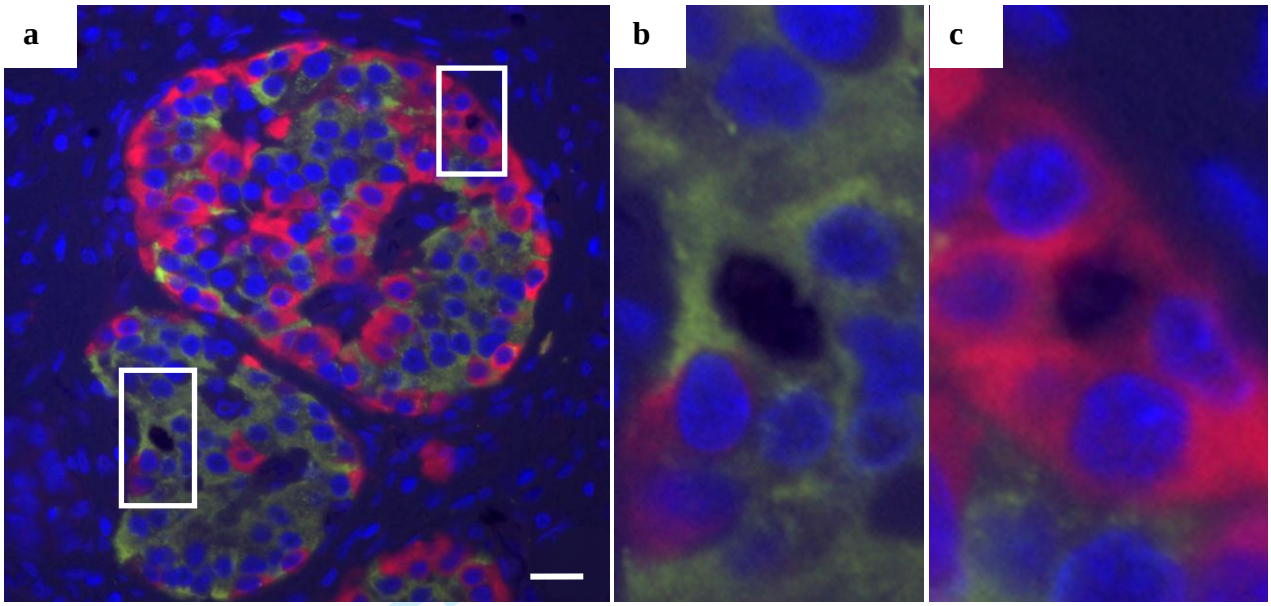


Figure 6.



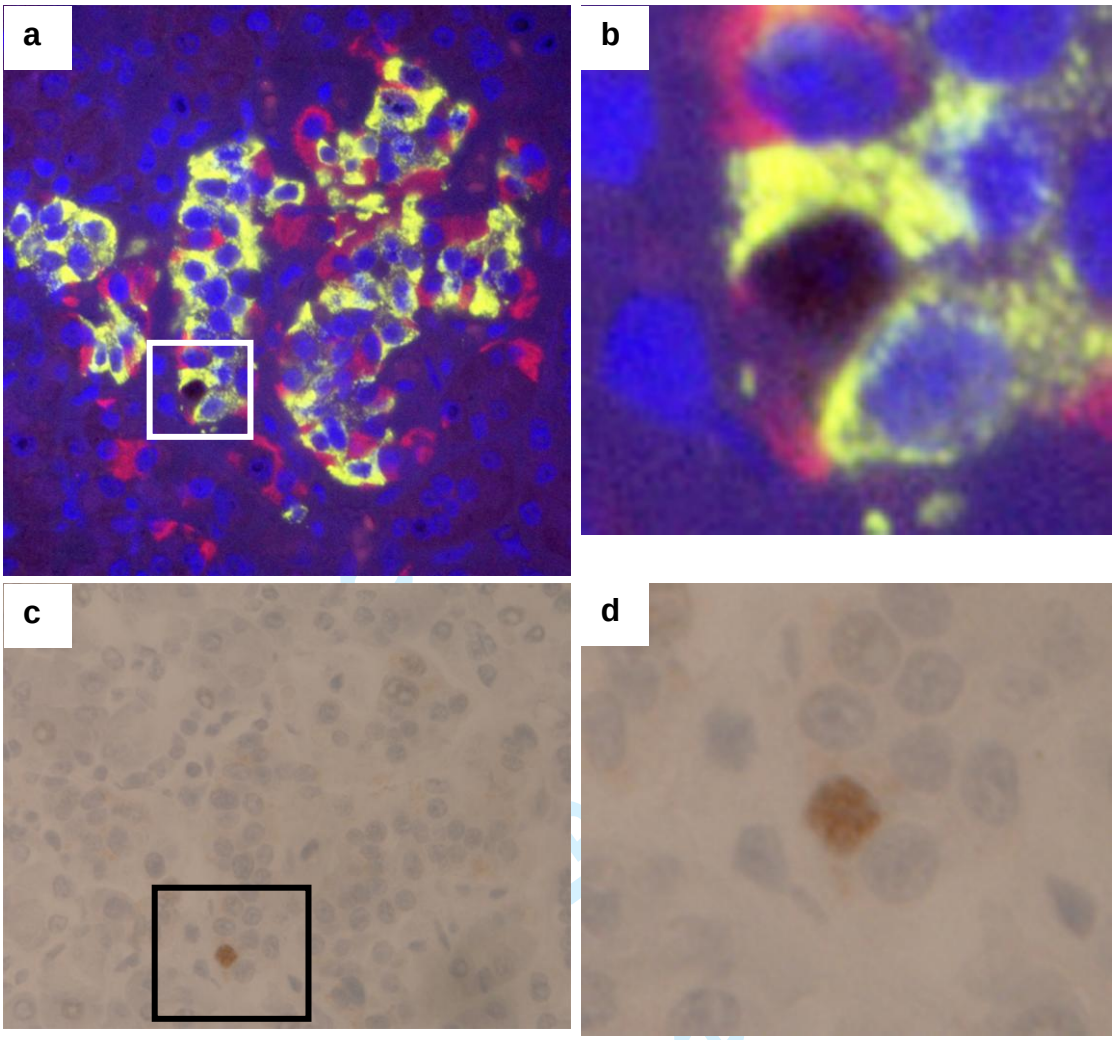
Electronic supplementary material Table 1 Details of control patients

Patient	Age	Gender	Illness	Cause of death (if applicable)	No. Islets	No. Ki67+ islets
C1	2	F		Bronchopneumonia and epilepsy	403	6
C2	2	M		Haemophilus influenzae meningitis	303	5
C3	3	F		Road traffic accident	337	4
C4	3	M		Meningococcal septicaemia	276	1
C5	3	F			277	2
C6	4	F			390	0
C7	5	M		Mental handicap	325	1
C8	6	M		Meningitis following head injury	270	9
C9	6	M		Porencephaly and pneumonia	295	8
C10	11	M			302	2
C11	38	M	Carcinoma of ampulla of Vater		98	2
C12	40	M	Insulinoma		181	1
C13	50	F	Insulinoma		345	0
C14	53	F	Insulinoma		229	1
C15	70	F	Type 2 diabetes		122	0
C16	72	F	Type 2 diabetes		164	1
C17	75	F	Type 2 diabetes		74	0
C18	75	F	Type 2 diabetes		170	2
C19	46	F	Type 2 diabetes	Hypoglycaemia	133	0
C20	56	M	Type 2 diabetes		70	2
C21	64	F	Type 2 diabetes	Following cholecystectomy	161	3
C22	71	M	Type 2 diabetes	Leg gangrene	169	1
C23	71	F	Type 2 diabetes	Myocardial infarction	224	2
C24	73	M	Type 2 diabetes	Meningitis	149	10
C25	20	M	Type 1 diabetes (duration 6years)		106	0
C26	22	M	Type 1 diabetes (duration 12years)		376	0
C27	25	F	Type 1 diabetes (duration 12years)	Pulmonary hypertension	79	0
C28	40	M	Chronic pancreatitis		247	5
C29	65	M	Chronic pancreatitis		144	0
C30	Unknown	M	Chronic pancreatitis		67	0
C31	Unknown	M	Chronic pancreatitis		762	7
C32	33	F	Chronic pancreatitis		100	16
C33	35	M	Chronic pancreatitis		577	21
C34	49	M	Chronic pancreatitis		127	0
C35	53	F	Chronic pancreatitis		389	10
C36	55	M	Chronic pancreatitis		137	4
C37	27	M	Autoimmune pancreatitis		151	9
C38	55	F	Autoimmune pancreatitis		180	7

C39	65	M	Chronic obstructive pancreatitis		108	3
C40	52	F		Perforated bowel cancer	42	0
C41	63	F		Subarachnoid	565	0.7
C42	63	M		Lung cancer	302	2.9
C43	68	M		Ruptured oesophagus	439	2.9
C44	68	M		Lung cancer	438	0.7
C45	69	F		Gall bladder cancer	311	0.3
C46	71	M		Lung cancer	435	0.2
C47	76	M		Stroke	300	0.6
C48	78	F		Ascending cholangitis	246	0.4

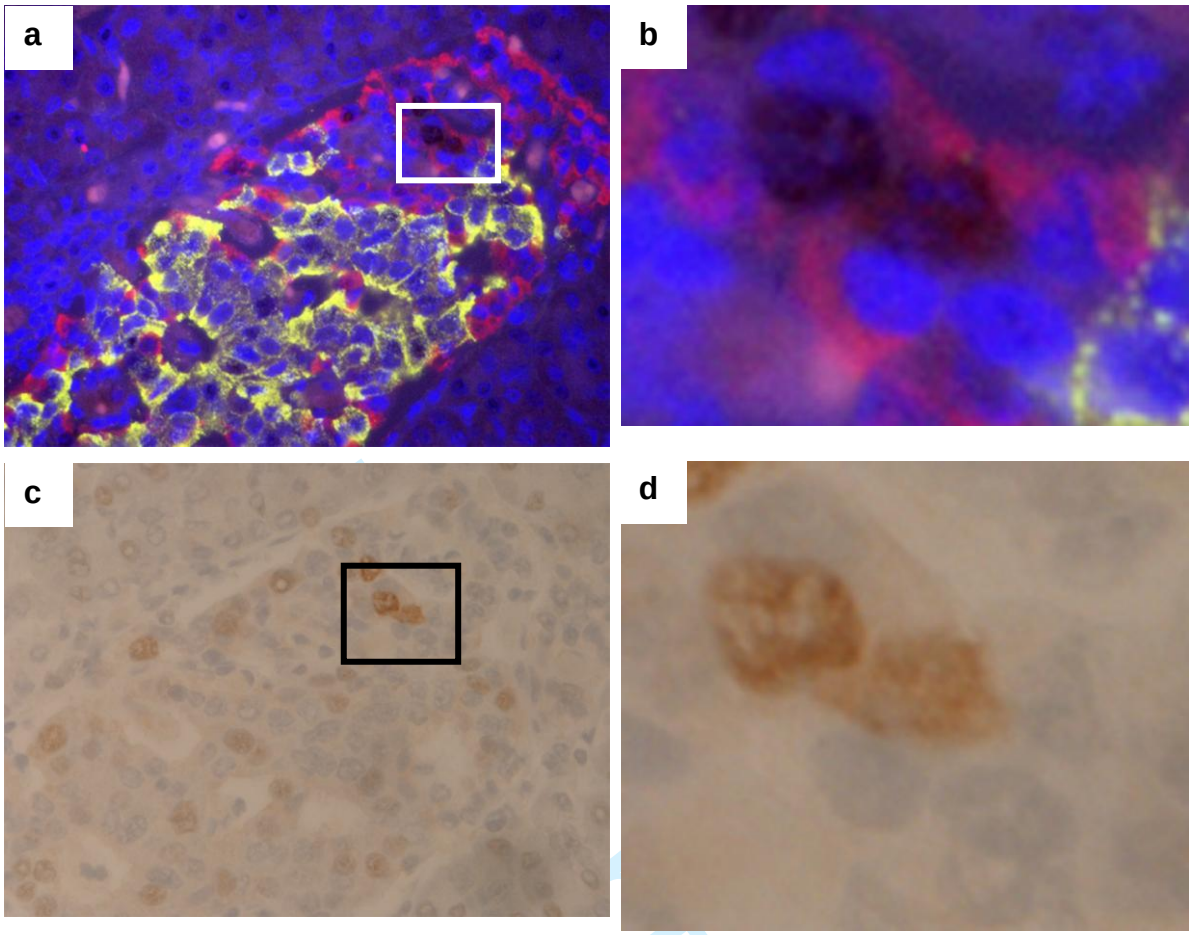
Note: In patients with pancreatitis (C28-C39) the majority (~ 70%) of Ki67+ cells detected in islets did not stain positively for insulin or glucagon.

Electronic Supplementary Material 2. Extended image plate Ki67+ Insulin+ cells



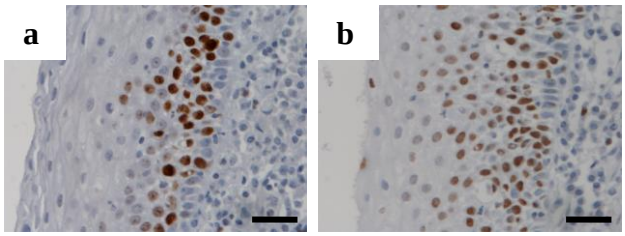
ESM 2. Photomicrographs of islets from type 1 diabetes patients stained using the combination technique to determine the cell type of the Ki67+ nuclei. (**a**) An islet showing insulin (green), glucagon (red) and Ki67 (dense black nuclei). The white box highlights the area focused on in image (**b**) which demonstrates a Ki67+, insulin+ cell indicative of a proliferating beta cell. (**c**) A brightfield image of the islet demonstrated in (**a**) in which the Ki67+ nucleus is shown in brown and the tissue is counterstained with hematoxylin (purple). The black box highlights the area focused on in image (**d**).

Electronic Supplementary Material 3. Extended image plate Ki67+ glucagon+ cells



ESM 3. Photomicrographs of islets from type 1 diabetes patients stained using the combination technique to determine the cell type of the Ki67+ nuclei. (**a**) An islet showing insulin (green), glucagon (red) and Ki67 (dense black nuclei). The white box highlights the area focused on in image (**b**) which demonstrates a Ki67+ glucagon+ cell indicative of a proliferating alpha cell. (**c**) A brightfield image of the islet demonstrated in image (**a**) which shows Ki67+ nuclei in brown and counterstaining with hematoxylin (purple). The black box highlights the area focused on in image (**d**).

Electronic Supplementary Material 4. Ki67 and Mcm-2 on tonsil epithelium



Mcm-2 is expressed for a longer period of time during the cell cycle than Ki67. Images (**a**) and (**b**) show Ki67+ and Mcm-2+ cells of the squamous epithelium of the tonsil respectively. Scale bar = 30 µm