A MEN1 pancreatic neuroendocrine tumour mouse model under temporal control

Endocrine CONNECTIONS

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Abstract

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterised by occurrence of parathyroid tumours and neuroendocrine tumours (NETs) of the pancreatic islets and anterior pituitary. The *MEN1* gene, encoding menin, is a tumour suppressor, but its precise role in initiating *in vivo* tumourigenesis remains to be elucidated. The availability of a temporally controlled conditional MEN1 mouse model would greatly facilitate the study of such early tumourigenic events, and overcome the limitations of other *MEN1* knockout models, in which menin is lost from conception or tumour development occurs asynchronously. To generate a temporally controlled conditional mouse model, we crossbred mice with the MEN1 gene floxed by LoxP sites (*Men1$^{L/L}$*), and mice expressing tamoxifen-inducible Cre recombinase under the control of the rat insulin promoter (*RIP2-CreER*), to establish a pancreatic β-cell-specific NET model under temporal control (*Men1$^{L/L}$/RIP2-CreER*). *Men1$^{L/L}$/RIP2-CreER* mice aged ~3 months were given tamoxifen in the diet for 5 days, and pancreata harvested 2–2.5, 2.9–3.5 and 4.5–5.5 months later. Control mice did not express *Cre* and did not receive tamoxifen. Immunostaining of pancreata from tamoxifen-treated *Men1$^{L/L}$/RIP2-CreER* mice, compared to control mice, showed at all ages: loss of menin in all islets; increased islet area (>4.2-fold); increased proliferation of insulin immunostaining β-cells (>2.3-fold) and decreased proliferation of glucagon immunostaining α-cells (>1.7-fold). There were no gender and apoptotic or proliferation differences, and extra-pancreatic tumours were not detected. Thus, we have established a mouse model (*Men1$^{L/L}$/RIP2-CreER*) to study early events in the development of pancreatic β-cell NETs.

Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder, characterised by the combined occurrence of tumours of the parathyroid glands, and neuroendocrine tumours (NETs) of the pancreatic islets and anterior pituitary (1). MEN1 is caused by heterozygous germline mutations of the *MEN1* gene, and tumours developed by MEN1 patients show loss of the remaining normal copy of the *MEN1* gene, a 'second-hit', demonstrating the tumour suppressor function of its ubiquitously expressed encoded protein, menin (2, 3, 4).

In MEN1 patients, ~50–70% of deaths are directly related to MEN1 syndrome with one of the most common causes of mortality being pancreatic neuroendocrine tumours (PNETs), which are frequently diagnosed at a metastatic stage and not curable by surgery (5, 6, 7, 8). In addition, over 40% of sporadic (non-familial) PNETs also show mutations in the *MEN1* gene (8, 9). Thus, *MEN1* mutations are frequently associated with PNET development. However, the *in vivo* mechanisms through which *MEN1* mutations initiate tumourigenic events are yet to be

fully elucidated. *In vitro* studies have shown that menin is a scaffold protein, which is able to bind a number of different proteins, and thereby exert its effects on multiple cellular mechanisms including epigenetic modification, transcriptional regulation, cell signalling and cell cycle regulation (5, 10). For example, in insulinoma cells, menin has been shown to bind to the mixed lineage leukaemia protein 1 (MLL1) to regulate histone methylation (5, 11), to inhibit cell cycle progression through interaction with cyclin-dependent kinase inhibitors (12) and to promote apoptosis through interaction with caspase 8 (13).

To facilitate *in vivo* studies several MEN1 mouse models have been generated and investigations of these have yielded important insights in NET cell proliferation and responses to treatments. For example, conventional heterozygous germline *Men1*-knockout mouse models, which develop parathyroid tumours and pancreatic and pituitary NETs and are representative of MEN1 in man, as well as tissue-specific conditional *Men1*-knockout mice, have been used to study tumour proliferation and the anti-proliferative effects of therapies including somatostatin analogues, growth factor receptor antibodies, tyrosine kinase inhibitors, wnt pathway modulators and *Men1* gene therapy (14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24). However, the dependence on the spontaneous occurrence of the second *Men1* mutation in the conventional *Men1* mouse model leads to a large variation in tumour type and age of occurrence, thereby limiting mechanistic studies aimed at determining the early tumourigenic events in specific tumours (14, 15, 16, 17). The use of conditional *Men1* mouse models helps to overcome the variation in tumour types as these MEN1 mice can be engineered to develop tumours in specific tissues. This is because the conditional *Men1*-knockout mice can be developed with tissue-specific homozygous deletion of the *Men1* alleles, using a site-specific recombination system in which genomic regions, flanked by LoxP sites, can be deleted using Cre recombinase (Cre-LoxP), and in which Cre acts under the influence of a tissue-specific promoter (25, 26). This has led to the development of mouse models in which pancreatic β-cell-specific rat insulin promoters are used to target Cre expression (*RIP-Cre*) and produce mice harbouring PNETs that express insulin (27, 28 29, 30). However, there is some leakage of Cre expression in the pituitary, and this results in the occurrence of prolactinomas in some of the *Men1*-knockout mice (28, 29). In addition, a mouse model in which a *RIP-Cre* promoter was used, developed PNETs expressing glucagon that is usually expressed in pancreatic α-cells, as well as PNETs expressing insulin (30).

Although these conditional models allow investigations of specific NET subtypes, their use for studying early tumourigenic events is limited by a lack of temporal control of the knockout event. Thus, in man, loss of menin usually occurs during adulthood and not during early development of the endocrine organs (1), which is the situation in the conditional *Men1* mice that have loss of menin expression from conception in the pancreatic β-cells, thereby precluding studies of early tumourigenic mechanisms that may occur rapidly after menin loss (27, 28, 29). Adding a temporal control to conditional models would help to overcome such limitations, as illustrated by use of a tamoxifen-inducible knockout of the *Men1* gene, activated by fusing a pan-active human ubiquitin C9 (UBC9) promoter to Cre recombinase and a modified oestrogen receptor (ER), to create a construct expressing tamoxifen inducible Cre recombinase under the control of the UBC9 promoter (*UBC9-Ert-Cre*) (31). These *Men1L/L/UBC9-Ert-Cre* conditional knockout mice developed, within 4 weeks of tamoxifen administration, islet hyperplasia with increased proliferation of insulin-expressing cells (31). However, the UBC9 promoter targets the whole pancreas and not a specific cell type, and although the UBC9-CreER complex successfully initiates *Men1* knockout in pancreatic islet cells of mice, it has not been reported to lead to pancreatic NET development (31). Such temporally controlled tissue-specific *Men1* knockout models could pave the way to exploration of the *in vivo* early molecular and cellular changes that occur after deletion on the *Men1* alleles. We therefore established a tamoxifen inducible *Men1L/L/RIP2-CreER* mouse model, with the aim of assessing its use as a pancreatic β-cell specific tumour model.

Materials and methods

Mouse breeding and genotyping

Animal studies were approved by the University of Oxford Ethical Review Committee and were licensed under the Animal (Scientific Procedures) Act 1986, issued by the United Kingdom Government Home Office Department (PPL30/2914). Stock Tg(Ins2-cre/ERT)1Dam/J (*RIP2-CreER*) and 129S(FVB)-Men1tm1.1Ctre/J (*Men1L/L*) mice were purchased from the Jackson Laboratory. *RIP2-CreER* mice, which express a pancreatic β-cell targeting promoter under the regulation of a tamoxifen inducible element (32), and *Men1L/L* mice, which contain the MEN1 gene with exons 3 and 8 floxed by loxP sites (28, 33), were

cross-bred to generate *Men1^{L/L}/RIP2-CreER* mice. *Men1^{L/L}/RIP2-CreER* mice were subsequently interbred and maintained on a mixed C57Bl/6 and 129S background. The mice were fed a standard diet (Rat and Mouse No. 1 expanded diet, Special Diet Services Ltd), provided with water *ad libitum*, and weighed regularly. Genotyping was performed using the following primers: *RIP2-CreER* (forward 1 5′-AAC CTG GAT AGT GAA ACA GGG GC-3′ and reverse 1 5′-TTC CAT GGA GCG AAC GAC GAG ACC-3′ and – forward 2 5′-CAA ATG TTG CTT GTC TGG TG-3′ and reverse 2 5′-GTC AGT CGA GTG CAC AGT TT-3′); and *Men1^{L/L}* (forward 1 5′-TCC AGT CCC TCT TCA GCT TC-3′ and reverse 1 5′-GCC ATT TCA TTA CCT CTT TCT CCG-3′ and reverse 2 5′-TAC CAC TGC AAA GGC CAC GC-3′). PCR reactions were performed as previously described (24).

Mouse phenotyping

Men1^{L/L}/RIP2-CreER mice were studied for the development of tumours at 2.0–2.5, 2.9–4.5 and 5.5–6.5 months (Table 1) after administration of oral tamoxifen, which commenced at ~3 months of age when mice had reached adulthood. Control, littermate, mice were also examined at the same time points (Table 1), which were chosen as they represent young adult and mature adult mice. Tamoxifen containing food pellets (1.2 g tamoxifen per kg of diet, Special Diet Services Ltd) were fed to mice, aged approximately 3 months of age, for five days in total, using a 2 days on, 1 day off (fed normal diet), 2 days on regime, before diet was returned to normal, as described previously (31). Mice are expected to eat approximately 11.1–15.6 g/day/100 g body weight of food pellets, which would yield a dose of approximately 200 mg/kg of tamoxifen per mouse, per day (34). Mice were weighed daily during this period to ensure diet consumption and that body weight was maintained. In addition, for 15–36 days prior to killing, mice were given drinking water that contained 1 mg/mL 5-bromo-2-deoxyuridine (BrdU), to allow assessment of cell proliferation, as previously described (23, 24, 35). In total, 46 mice (22 males and 24 females) were studied, and comprised 26 *Men1^{L/L}/RIP2-CreER* mice that received tamoxifen treatment (treated group), and 20 control mice (Table 1), which consisted of *Men1^{L/L}/RIP2-CreER* mice maintained on a normal diet or *Men1^{L/L}* mice not expressing the *RIP2-CreER* allele, but fed tamoxifen. The mice were culled at 5.0–5.5, 5.9–6.5 or 7.5–8.5 months of age and weighed, and a full necropsy was performed to harvest the pancreas, which was fixed in 4% paraformaldehyde and embedded in paraffin, as previously described (23, 24, 35).

Histological analysis

Paraffin-embedded serial sections (5 μm) of the pancreata were prepared, using a microtome (Leica RM 2255), dewaxed, hydrated and treated for antigen retrieval at 120°C in a citrate buffer solution (pH 6), before blocking in 10% donkey serum, as previously described (24, 35). From each pancreas, four sections were stained for menin, and two sections for chromogranin A, insulin and glucagon using the diaminobenzidine (DAB) kit (Dako) and counterstained with haematoxylin, as previously described (16, 35). Two additional sections from each pancreas were also fluorescently co-stained with BrdU and insulin, or BrdU and glucagon, as previously described (23, 24, 35), using appropriate primary and secondary antibodies (Table 2). DAB and fluorescently stained sections were mounted using Vectamount permanent mounting medium (Vector labs) or ProLong Gold antifade reagent with DAPI (Life Technologies), respectively. Sections were viewed by light or fluorescent microscopy using an Eclipse E400 microscope (Nikon), and images were captured using a DXM1200C digital camera and NIS-Elements BR 2.30 software (both Nikon). Three islets per section were analysed, yielding a total of 12 islets for menin expression and 6 islets for immunostaining studies, for each mouse.

Menin, insulin and glucagon expression was analysed qualitatively, by two independent investigators. Serial sections were analysed to allow for detection of tumours expressing insulin or glucagon. Islet area (μm²)

Table 1 Numbers, age (in months) and gender of control and tamoxifen-treated (treated) groups.

Age	Group	
	Control	Treated
5.0–5.5	*N* = 6 (3 male, 3 female)	*N* = 8 (3 male, 5 female)
5.9–6.5	*N* = 8 (4 male, 4 female)	*N* = 11 (5 male, 6 female)
7.5–8.5	*N* = 6 (3 male, 3 female)	*N* = 7 (4 male, 3 female)
Total	*N* = 20 (10 male, 10 female)	*N* = 26 (12 male, 14 female)

Table 2 Antibodies used in the immunostaining protocols.

Immunostaining target (sections per pancreas)	Primary antibody (RRID)	Secondary antibody (RRID)
Menin (4)	Rabbit anti menin (AB_303203)*	Horseradish peroxidase conjugated donkey anti-rabbit (AB_10015282)**
Chromogranin A (2)	Rabbit anti chromogranin A (AB_301704)	Horseradish peroxidase conjugated donkey anti-rabbit (AB_10015282)**
Glucagon (2)	Rabbit anti glucagon (AB_10561971)*	Horseradish peroxidase conjugated donkey anti-rabbit (AB_10015282)**
Insulin (2)	Guinea pig anti insulin (AB_306130)*	Horseradish peroxidase conjugated rabbit anti guinea pig***
BrdU/insulin (2)	Rat anti BrdU (AB_305426)* Guinea pig anti insulin (AB_306130)*	Donkey anti rat Cy3 (AB_2340667)** Donkey anti guinea pig Cy2 (AB_2340467)**
BrdU/glucagon (2)	Rat anti BrdU (AB_305426)* Rabbit anti glucagon (AB_10561971)*	Donkey anti rat Cy3 (AB_2340667)** Donkey anti rabbit Alexa Fluor 488 (AB_2535792)****

*Abcam; **Jackson ImmunoResearch; ***Dako (Agilent Technologies); ****Thermo Fischer scientific.

was measured in menin stained sections using the area selection tool in NIS-Elements BR 2.30 software (Nikon). The mean islet area per pancreas, per mouse was calculated.

The proliferation rates of insulin-expressing and glucagon-expressing cells were determined by counting the number of BrdU-positive cells relative to the total number of insulin- or glucagon-expressing cells, adjusted for the length of BrdU exposure using the following formula: ((BrdU positive insulin/glucagon-expressing cells)/(total insulin/glucagon-expressing cells)/(days of BrdU administration)*100), as previously described (35). In total $n=12$ islets or tumours, from 4 sections were counted per mouse and the mean value per pancreas, per mouse was calculated. BrdU is incorporated into any proliferating cell and to ensure that only islet cells were quantified in our study, we included only those cells co-stained for either insulin or glucagon, thereby excluding leukocytes, fibroblasts and exocrine pancreatic cells, which were identified in control and treated mice of all age groups.

Apoptosis was evaluated using the ApopTag Fluorescein Direct *In Situ* Apoptosis Detection Kit (Millipore), which utilises terminal deoxynucleotidyl transferase (TdT) to detect and label free 3′OH DNA termini caused by fragmentation, as previously described (23, 24, 35). Apoptotic rate was determined by counting the number of apoptotic cells per islet/tumour area, from a total of 16 islets (4 islets from 4 sections). The mean value per pancreas, per mouse was calculated.

Statistical analysis

GraphPad Prism was used for the statistical analyses and the generation of graphs. A *t*-test, corrected for multiple testing using the Bonferroni correction, was used to test for significance of results, with the threshold of significance being set at $P<0.05$, as previously described (23, 24, 35).

Results

Occurrence of pancreatic islet NETs in *Men1^{L/L}/RIP2-CreER* mice, and expression of menin, insulin and glucagon

Macroscopic and microscopic (Fig. 1) examination identified tumours of the pancreas in 100% of male and female *Men1^{L/L}/RIP2-CreER* mice fed tamoxifen (treated group, $n=26$), at all time points, with no tumours identified in the control group ($n=20$). Furthermore, macroscopic examination did not identify tumours in any other organs, including the pituitary or any metastatic lesions, including in the liver or lymph nodes. Despite the occurrence of the pancreatic tumours, the tamoxifen-treated group of mice appeared healthy with similar body weights (Fig. 2) and had no increase in mortality, when compared to the control group of mice. Moreover, microscopy of the pancreatic islet revealed that the tamoxifen-treated *Men1^{L/L}/RIP2-CreER* mice had significantly larger islets than the control mice (Figs 1 and 3). Analysis of these data by gender did not reveal any significant differences between males and females in any age group (Fig. 3A, B and C), and therefore, statistical analysis was performed on combined male and female data. Thus, the tamoxifen-treated *Men1^{L/L}/RIP2-CreER* mice, when compared to control mice, had significantly larger mean islet areas (islets of tamoxifen-treated mice were larger by 4.3-fold ($P<0.005$), 4.2-fold ($P<0.0005$) and 4.2-fold ($P<0.0005$) at 5.0–5.5 months
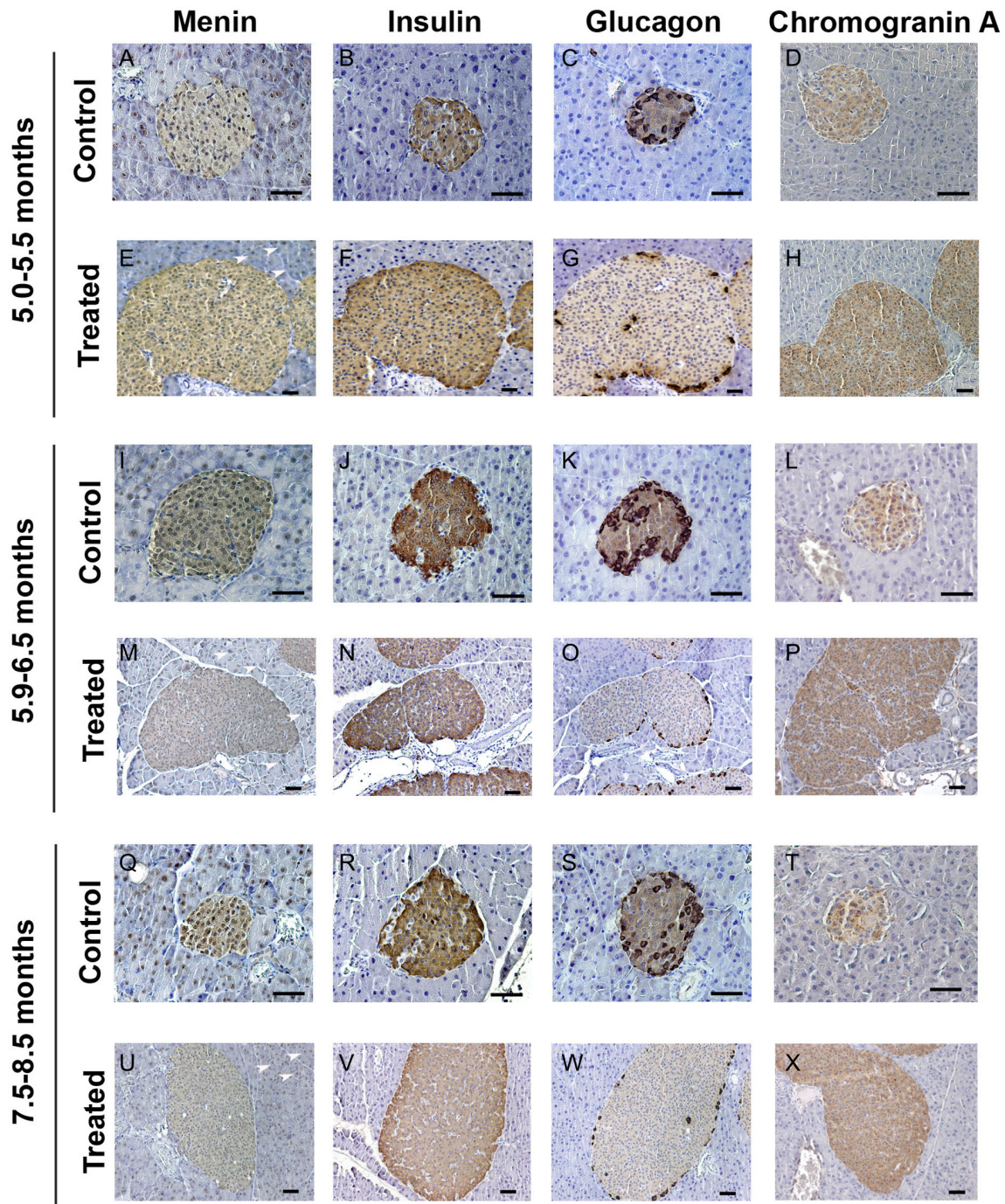
Figure 1

Expression of menin (A, E, I, M, Q, U), insulin (B, F, J, N, R, V), glucagon (C, G, K, O, S, W) and chromogranin A (D, H, L, P, T, X) in pancreatic islets of control or tamoxifen-treated (Treated) *Men1^L/L^/RIP2-CreER* mice. Expression (brown) was detected by immunohistochemical staining, and sections were counterstained with haematoxylin (blue). Control sample images (A–D, I–L, Q–T) are at ×40 magnification, and tamoxifen-treated sample images (E–H, M–P, U–X) are at ×20 magnification, with the scale bar representing 50 µm. Representative images are shown from female mice. Similar results were observed in male mice (data not shown). The ages of the treated (*Men1^L/L^/RIP2-CreER* mice treated with tamoxifen at ~3 months of age) and control (*Men1^L/L^/RIP2-CreER* mice without treatment) groups are shown in months. Retained menin expression in exocrine pancreas of treated mice is indicated by white arrows.

($n = 6$ control and $n = 8$ treated), 5.9–6.5 months ($n = 8$ control and $n = 11$ treated) and 7.5–8.5 months ($n = 6$ control and $n = 7$ treated), respectively, when compared to controls) (Fig. 3D), consistent with tumour development in all the age groups. Moreover, immunohistochemistry analysis of the pancreatic sections from tamoxifen-treated
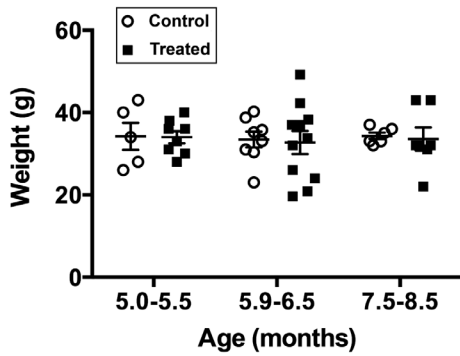
Figure 2

Body weights of control and tamoxifen-treated (treated) *Men1^{L/L}/RIP2-CreER* mice at the end of the study. Control mice are represented as white circles and tamoxifen-treated (treated) *Men1^{L/L}/RIP2-CreER* mice as black squares. Significant differences were not observed in the body weight of control or tamoxifen-treated mice in any age group. Data are represented as the mean and s.e.m.

Men1^{L/L}/RIP2-CreER mice revealed a loss of nuclear menin expression, in contrast to the observed expression of menin in the nucleus of >95% of pancreatic islets cells in control mice (Fig. 1A, E, I, M, Q and U). In contrast, nuclear menin staining of both control and tamoxifen-treated mice was detected in exocrine pancreatic cells,

and this acted as a positive control for menin staining and confirmed that the ER-driven Cre expression was limited to pancreatic islet cells (Fig. 1A, E, I, M, Q and U). Immunohistochemical analysis of hormone expression indicated that control islets and tumours of *Men1^{L/L}/RIP2-CreER* tamoxifen-treated mice expressed the endocrine marker chromogranin A, thereby confirming that the tumours are arising from the neuroendocrine cells (Fig. 1D, H, L, P, T and X). However, control islets expressed a normal murine hormonal expression pattern with predominant expression of insulin (Fig. 1B, J and R), and with glucagon expression in cells located around the periphery of the islet (Fig. 1C, K and S) (23, 24, 35). In contrast to hormonal expression in islets of control mice, islets of all tamoxifen-treated mice mostly expressed insulin (Fig. 1F, N and V), with scanty and peripheral expression of glucagon (Fig. 1G, O and W). Moreover, the glucagon-expressing cells retained menin expression, thereby indicating that they were likely residual non-tumourigenic glucagon-expressing α-cells. These findings that were similar in male and female mice, along with our data indicating that there is a relative increase of insulin-expressing cells (Fig. 3E) in tamoxifen-treated mice older than 5.0–5.5 months but no increase in the number of
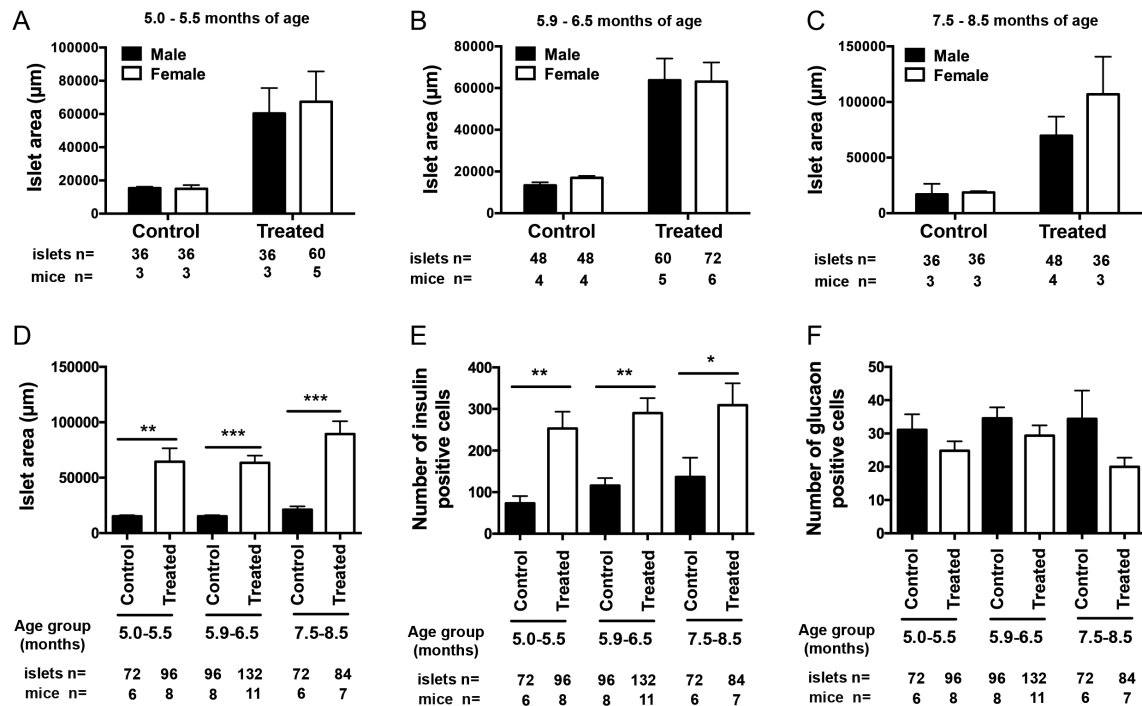

Figure 3

Pancreatic islet area of *Men1^{L/L}/RIP2-CreER* mice treated with tamoxifen (treated) and control groups. Pancreatic islet area (μm²) of male and female mice aged 5.0–5.5 months (A), 5.9–6.5 months (B) and 7.5–8.5 months (C) and the total group of *n* = 46 (males = 22 and females = 24) (D) are shown. In addition, quantification of insulin (E) and glucagon (F) immunostaining cells is shown. Four sections and 3 islets per section were analysed from each mouse. Data are represented as the mean and s.e.m. *P < 0.05; **P < 0.005; ***P < 0.0005.

glucagon-expressing cells (Fig. 3F), is consistent with tumour development originating from β-cells.

Proliferation and apoptosis rates of islet cells

Analysis of the proliferation rates of pancreatic islets from the control and tamoxifen-treated *Men1$^{L/L}$/RIP2-CreER* mice revealed that the proliferation rate of insulin-expressing cells in tamoxifen-treated mice was significantly higher than that in the control mice (Fig. 4A and B), with no gender differences observed. Thus, the mean proliferation rate in tamoxifen-treated mice when compared to control mice was 3.9-fold greater at 5.0–5.5 months ($P < 0.005$), 2.6-fold greater at 5.9–6.5 months ($P < 0.005$) and 2.3-fold greater at 7.5–8.5 months ($P < 0.005$) (Fig. 4B). Interestingly, the proliferation rate of insulin-expressing cells in the 5.0- to 5.5-month-old tamoxifen-treated mice was significantly higher than that of the tamoxifen-treated mice in the 5.9- to 6.5-month and 7.5- to 8.5-month age groups, by 1.9-fold ($P < 0.0005$) and 1.7-fold ($P < 0.05$), respectively. The proliferation rates of insulin-expressing cells in the

tamoxifen-treated mice aged 5.9–6.5 and 7.5–8.5 months were not significantly different, and the proliferation rates of insulin-expressing cells in all of the control groups were similar (Fig. 4B). Proliferation rates of glucagon-expressing α-cells in the tamoxifen-treated *Men1$^{L/L}$/RIP2-CreER* mice (Fig. 5A and B) showed that these α-cells, which retained menin expression, at 5.9–6.5 and 7.5–8.5 months of age were lower when compared to those of control mice. Thus, in the 5.9–6.5 and 7.5–8.5-month-old mice, mean proliferation rates were significantly lower, in the tamoxifen-treated group, by 2-fold ($P < 0.005$) and 2.2-fold ($P < 0.05$), respectively (Fig. 5B) than those in the control group; no gender differences were observed. Of note, the proliferation rates of glucagon-expressing cells when compared to insulin-expressing cells were 6.4-fold lower in control mice and 31.2-fold lower in treated mice. The combined data from the α- and β-cells indicate that the increase in islet area is not the consequence of an increase in proliferation of glucagon-expressing cells, but is instead likely due to a markedly increased proliferation rate of insulin-expressing β-cells. Apoptosis rates were similar in tamoxifen-treated and control mice in all age
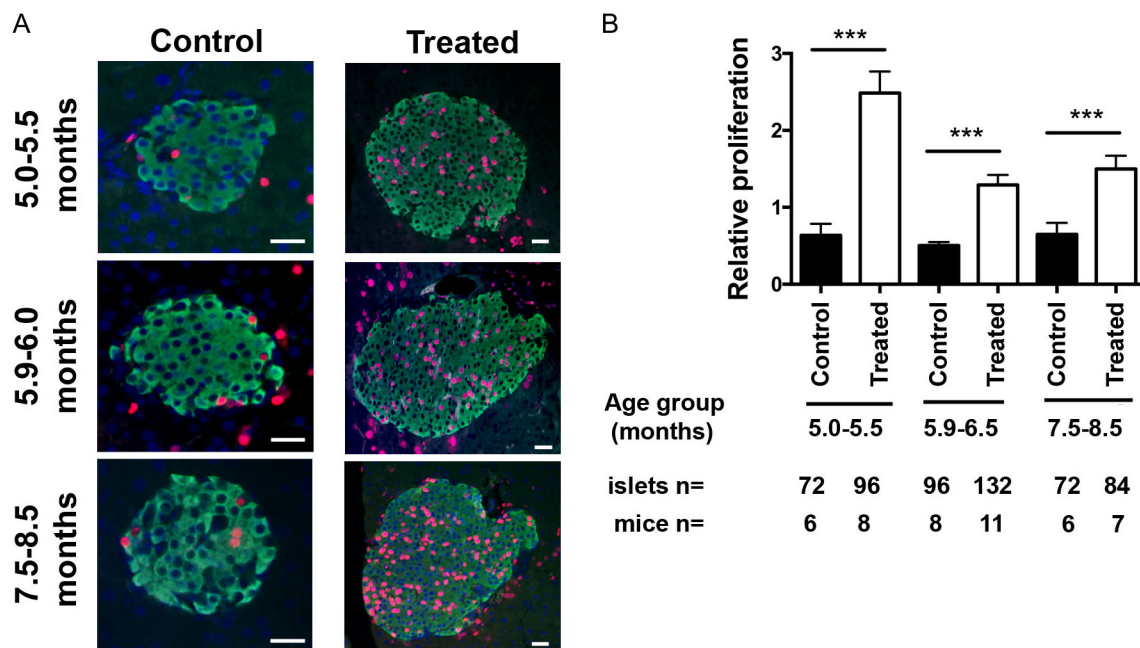

Figure 4

Proliferation of insulin-expressing pancreatic islet cells from control and tamoxifen-treated (treated) *Men1$^{L/L}$/RIP2-CreER* mice. Proliferation was assessed by nuclear incorporation of BrdU (red) in insulin (green) expressing cells, using fluorescent immunostaining; nuclei are indicated by DAPI staining (Blue) (A). The ages of the treatment (*Men1$^{L/L}$/RIP2-CreER* mice treated with tamoxifen at ~3 months of age) and control (*Men1$^{L/L}$/RIP2-CreER* mice without treatment) are shown in months, together with the numbers of mice and islets analysed in each group. Control images are at ×40 magnification, and treated images at ×20 magnification, with the scale bar representing 50 µm. Relative proliferation was quantified as the proportion of insulin-expressing cells immunostaining for BrdU, times the number of days of BrdU administration (B). Data is represented as the mean and s.e.m., ***$P < 0.005$. BrdU immunostaining cells that are adjacent to the islet tumours, but do not immunostain for insulin, are likely to be proliferating leukocytes, fibroblasts or exocrine pancreatic cells.
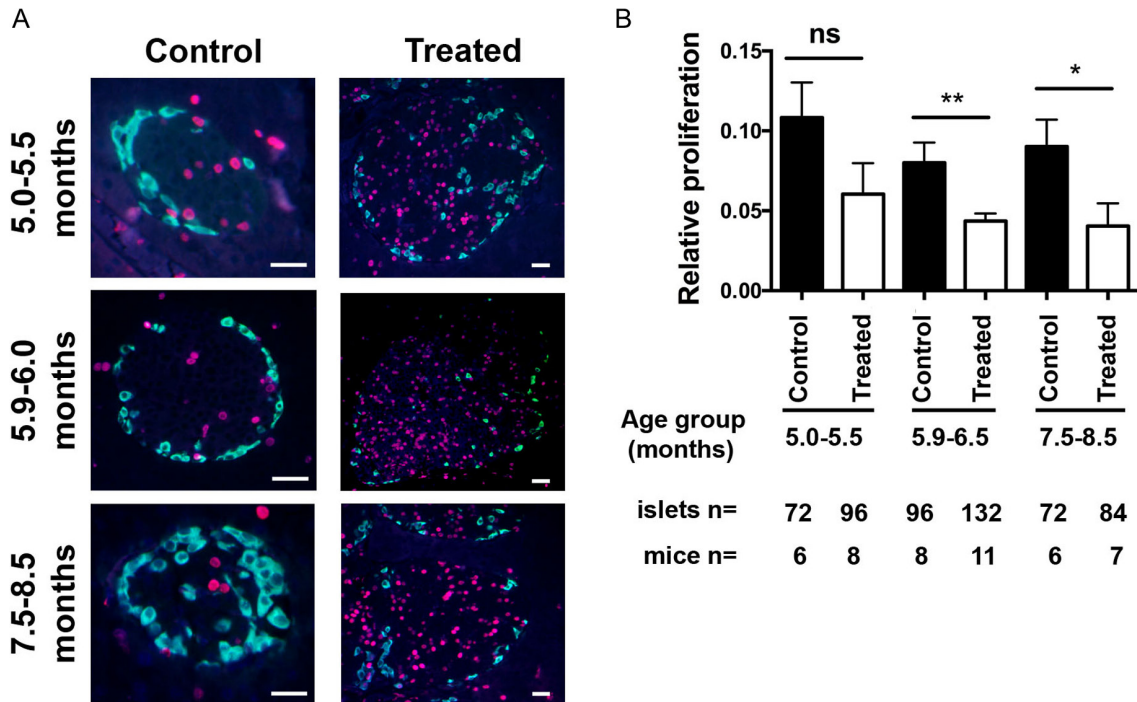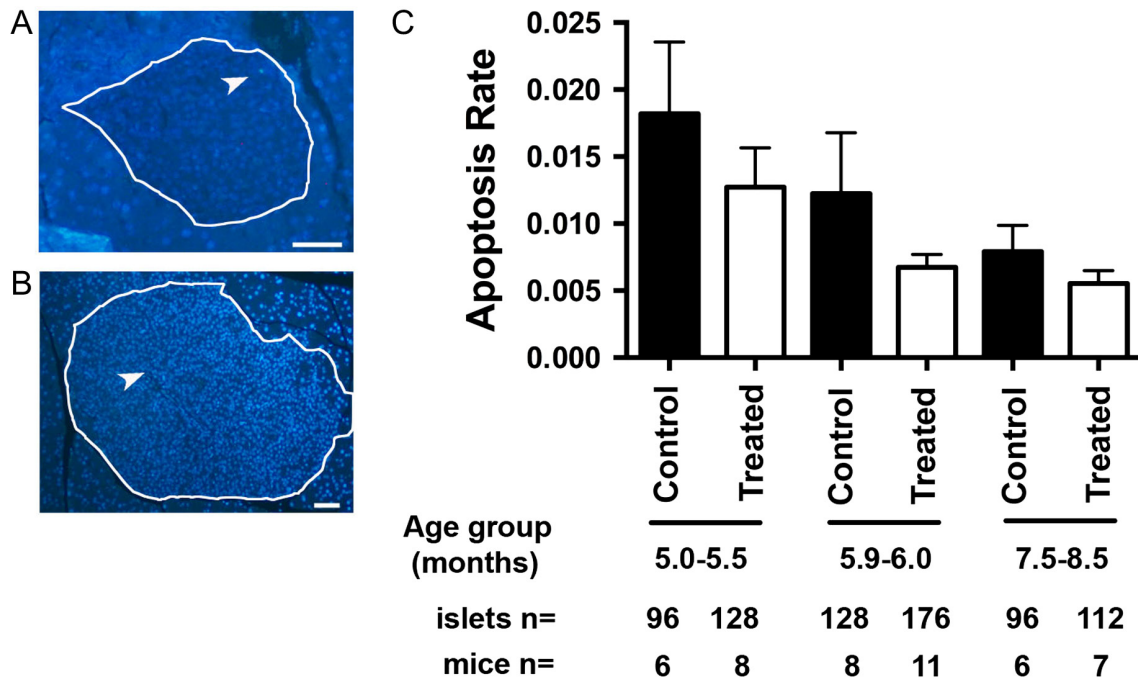
Figure 5

Proliferation of glucagon-expressing pancreatic islet cells from control and tamoxifen-treated (treated) *Men1*[L/L]/*RIP2-CreER* mice. Proliferation was assessed by nuclear incorporation of BrdU (red) in glucagon (green)-expressing cells, using fluorescent immunostaining; nuclei are indicated by DAPI staining (Blue) (A). The ages of the treatment (*Men1*[L/L]/*RIP2-CreER* mice treated with tamoxifen at ~3 months of age) and control (*Men1*[L/L]/*RIP2-CreER* mice without treatment) are shown in months, together with the number of mice and islets analysed in each group. Control images are at ×40 magnification, and test images at ×20 magnification, with the scale bar representing 50 μm. Relative proliferation was quantified as the proportion of glucagon-expressing cells that immunostained for BrdU, corrected for number of days of BrdU administration (B). Data are represented as the mean and s.e.m., *$P < 0.05$; **$P < 0.005$; ns, not significant. BrdU immunostaining cells that are adjacent to the islet tumours, but do not immunostain for glucagon, are likely to be proliferating leukocytes, fibroblasts or exocrine pancreatic cells.

groups (Fig. 6), and overall apoptosis rate was low in all age groups with only 0–5 apoptotic cells identified per islet (Fig. 6A and B).

Discussion

Our studies show that in *Men1*[L/L]/*RIP2-CreER* mice, loss of menin could be induced by administration of tamoxifen, leading to the development of pancreatic β-cell islet tumours. We chose to generate *Men1*[L/L]/*RIP2-CreER* mice, by intercrossing MEN1 LoxP floxed mice, with mice expressing the rat insulin 2 promoter, under the control of a tamoxifen inducible element (*RIP2-CreER*), rather than other pancreatic β-cell-specific targeting constructs such as the human insulin promoters *Ins1* and *Ins2*, and the rat insulin promoters *RIP1*, *2* and *7* (28, 36, 37) for the following reasons. Thus, an inducible *RIP2-Cre* construct was chosen for this study as the *RIP2-Cre* construct and the Cre-LoxP system have previously been used successfully to develop NET mouse models,

including those for pancreatic insulinomas (18, 26, 27, 28, 29). The disadvantage of the *RIP2-Cre* construct is that the Cre expression may occasionally occur in the pituitary (29, 36). However, for the investigation of MEN1 tumourigenesis, this is not necessarily a disadvantage, as MEN1 patients also develop pituitary tumours (5). Furthermore, the inappropriate Cre expression in the pituitary is reported to only occur in female mice (29, 36), thereby indicating that use of the *RIP2* promoter may provide a model by which two different tumour types can be examined in the same female mouse. However, in our study macroscopic examination of the pituitary did not identify any tumours, in female or male mice. In addition, it has been reported that glucagonomas may occur with use of the *RIP* promoter (30). This was not observed in our study, as all tumours predominantly expressed insulin (Fig. 1), and significant increases in proliferation were only observed in insulin- (β-cells), and not glucagon- (α-cells), expressing cells (Figs 4 and 5). In addition, macroscopic examination did not identify any metastatic lesions, for example in the liver,

Figure 6

Apoptosis in pancreatic islets from *Men1L/L/RIP2-CreER* mice treated with tamoxifen (treated groups) and control groups. Representative images from controls (A) ×40 magnification and tamoxifen-treated ×20 magnification (B) aged 5.9–6.5 months are shown; the scale bar represents 50 µm on both images. Islet area is indicated in white, apoptotic cells are represented in green (and indicated by a white arrow). Four islets from 4 sections from each mouse were assessed. Very few apoptotic cells were detected per islet. Quantification of apoptotic rates in mice aged 5.0–5.5, 5.9–6.5 and 7.5–8.5 months revealed there to be no significant differences in the rate of apoptosis in control and treated mouse groups (C). Data are represented as mean with s.e.m.

consistent with previous conditional and conventional *Men1*-knockout mouse models (16, 17, 28, 29, 31). Furthermore, our results showed that at 5.0–5.5 months of age the size of the islets from tamoxifen-treated *Men1L/L/RIP2-CreER* mice were 4.3-fold greater than those from control mice (Fig. 3), and that the proliferation rate of insulin-expressing cells in the tamoxifen-treated *Men1L/L/RIP2-CreER* mice was increased by 3.9 ± 0.44-fold when compared to control mice (Fig. 4). Thus, our findings suggest that the *Men1L/L/RIP2-CreER* model is a useful model for insulin-expressing NETs at 5.0–5.5 months of age, which is considerably less than that of the 12–18 months required for development of similar NETs in the conventional models (14, 15, 16, 17). Thus, our study helps to provide useful refinements in the methodology of animal experiments, as promoted by the National Centre for the Replacement, Refinement and Reduction of animals in research (NC3Rs) (38). Moreover, our study shows that drinking water and food pellets can be used to successfully deliver agents such as BrdU and tamoxifen, and thereby allow spatiotemporal control of tumour development, which may allow for a reduction in the numbers of mice required for studies

of tumourigenic mechanisms and therapies (14, 15, 16, 17, 39).

Our data demonstrate an increase in the number of insulin-expressing cells in treated, compared to control mice (Fig. 4A), without a change in the number of glucagon-expressing cells in tamoxifen-treated mice, compared to control mice (Fig. 3F), despite there being a significant decrease in proliferation of these cells (Fig. 5A). There are possible explanations for these findings, as follows. First, pancreatic α, β and δ cells have been reported to have the ability to transdifferentiate into different, functioning, pancreatic islet cell types under metabolic stress (40), and it may be that transdifferentiation is occurring in the tumourigenic islets, for example, δ cells transdifferentiating into α-cells, to attempt to compensate for the malfunctioning islet. Second, an intermediary stage of transdifferentitation is dedifferentiation, and it has been shown that dedifferentiation may also occur in islets under metabolic stress (40). Therefore, cells in tumourigenic islets may be undergoing dedifferentiation, although this possibility may be less likely, as in our studies all proliferating cells appeared to be expressing insulin or glucagon, with no evidence of hormone-negative proliferating

tumour cells. Further, detailed investigations would be required to determine whether transdifferentiation or dedifferentiation is occurring in these tumourigenic islets.

Our results reveal that menin loss is associated with a significant increase in the proliferation of β-cells, and this is consistent with the reports that menin dose is important for the regulation of proliferation (41) and that loss of menin expression results in pancreatic NETs in humans and mice (3, 8, 14, 15, 16, 18, 27, 28, 29, 30, 32, 36, 39, 42). Moreover, the observed greater increase in the proliferation rate of the younger 5.0- to 5.5-month-old mice, when compared to that in the 5.9- to 6.5- and 7.5- to 8.5-month-old mice (Fig. 4A), is consistent with menin loss being an initial driver of increased proliferation. However, our results also suggest that although menin loss may have provided the initial drive of proliferation in insulin-expressing cells that other alterations may also be required for tumours to progress and to become clinically aggressive (4, 42). These may include a limitation of nutrients as the tumour mass expands, that will slow proliferation rates, and further genetic alterations, for example, in genes associated with angiogenesis that may accelerate tumour progression. Furthermore, it has been reported that although MEN1 tumours develop in patients after loss of menin expression, that in mice with heterozygous MEN1 loss, there is a significant increase in proliferation of pancreatic islets, thereby indicating that menin dose may be important for the regulation of proliferation. The time-controlled loss of menin in the *Men1$^{L/L}$/RIP2-CreER* mice may also help to provide a useful model for the investigation of these early genetic and molecular mechanisms that may be occurring in pancreatic islet cells before, during and after menin loss. Thus, our findings show that *Men1$^{L/L}$* and *RIP2-CreER* constructs can be used to successfully knock out menin expression specifically in pancreatic β-cells, in a time-dependent manner.

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