A MEN1 pancreatic neuroendocrine tumour mouse model under temporal control

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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 \textit{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 (\textit{Men1\textsuperscript{L/L}}), and mice expressing tamoxifen-inducible Cre recombinase under the control of the rat insulin promoter (\textit{RIP2-CreER}), to establish a pancreatic \(\beta\)-cell-specific NET model under temporal control (\textit{Men1\textsuperscript{L/L}/RIP2-CreER}). \textit{Men1\textsuperscript{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 \textit{Men1\textsuperscript{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 \(\beta\)-cells (>2.3-fold) and decreased proliferation of glucagon immunostaining \(\alpha\)-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 (\textit{Men1\textsuperscript{L/L}/RIP2-CreER}) to study early events in the development of pancreatic \(\beta\)-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 \textit{MEN1} gene, and tumours developed by MEN1 patients show loss of the remaining normal copy of the \textit{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 \textit{MEN1} gene (8, 9). Thus, \textit{MEN1} mutations are frequently associated with PNET development. However, the \textit{in vivo} mechanisms through which \textit{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 mouse 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 (PL30/2914). 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<sup>+/−</sup>/RIP2-CreER mice. Men1<sup>+/−</sup>/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 <em>ad libitum</em>, 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 GTC TGG TG-3′ and reverse 2 5′-GTC AGT CGA GTG TT-3′); and Men1<sup>+/−</sup> (forward 1 5′-TCC AGT CCC TCT CTA CCG-3′ and reverse 1 5′-GCC ATT TCA TTA CCT CTT TCT CCG-3′ and reverse 2 5′-TAC CAC CAC TGC AAA GGC CAC GC-3′). PCR reactions were performed as previously described (24).

**Mouse phenotyping**

Men1<sup>+/−</sup>/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<sup>+/−</sup>/RIP2-CreER mice that received tamoxifen treatment (treated group), and 20 control mice (Table 1), which consisted of Men1<sup>+/−</sup>/RIP2-CreER mice maintained on a normal diet or Men1<sup>+/−</sup> 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)

| Age | Numbers, age (in months) and gender of control and tamoxifen-treated (treated) groups. |
|-----|--------------------------------------------------------------------------------------------------------------------------|
|     |                                                                                                                          |
| 5.0-5.5 | Numbers, age (in months) and gender of control and tamoxifen-treated (treated) groups.                                      |
| 5.9–6.5 | N=6 (3 male, 3 female)                                                                                                   |
| 7.5–8.5 | N=8 (4 male, 4 female)                                                                                                   |
| Total  | N=11 (5 male, 6 female)                                                                                                  |
| Group |                                                                                                                          |
| Control | N=26 (12 male, 14 female)                                                                                               |
| N=20 (10 male, 10 female)                                                                                               |
| N=6 (3 male, 3 female)                                                                                                   |
| N=8 (3 male, 5 female)                                                                                                   |
| N=7 (4 male, 3 female)                                                                                                   |
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 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 Men1L/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 Men1L/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 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 Men1L/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 Men1L/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

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)*  | Donkey anti rat Cy3 (AB_2340667)** |
| BrdU/glucagon (2)                            | Rat anti BrdU (AB_305426)*  | Donkey anti rat Cy3 (AB_2340667)** |

*Abcam; **Jackson ImmunoResearch; ***Dako (Agilent Technologies); ****Thermo Fischer scientific.
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) Men1L/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 (Men1L/L/RIP2-CreER mice treated with tamoxifen at ~3 months of age) and control (Men1L/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
Men1<sup>L/L</sup>/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). Immunohistochemical analysis of hormone expression indicated that control islets and tumours of Men1<sup>L/L</sup>/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 2**

Body weights of control and tamoxifen-treated (treated) Men1<sup>L/L</sup>/RIP2-CreER mice at the end of the study. Control mice are represented as white circles and tamoxifen-treated (treated) Men1<sup>L/L</sup>/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.

**Figure 3**

Pancreatic islet area of Men1<sup>L/L</sup>/RIP2-CreER mice treated with tamoxifen (treated) and control groups. Pancreatic islet area (μm<sup>2</sup>) 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<sup>−/−</sup>/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 mouse 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<sup>−/−</sup>/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](http://www.endocrineconnections.org)

**Figure 4**

Proliferation of insulin-expressing pancreatic islet cells from control and tamoxifen-treated (treated) Men1<sup>−/−</sup>/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<sup>−/−</sup>/RIP2-CreER mice treated with tamoxifen at ~3 months of age) and control (Men1<sup>−/−</sup>/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 50μm. 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.
Research

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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 Men1<sup>L/L</sup>/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 Men1<sup>L/L</sup>/RIP2-CreER mice was increased by 3.9 ± 0.44-fold when compared to control mice (Fig. 4). Thus, our findings suggest that the Men1<sup>L/L</sup>/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 transdifferentiation 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 cells.
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 Men1Δ/Δ/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 Men1Δ/Δ and RIP2-CreER constructs can be used to successfully knock out menin expression specifically in pancreatic β-cells, in a time-dependent manner.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References
1 Thakker RV, Newey PJ, Walls GV, Bilezikian J, Drake H, Ebeling PR, Melmed S, Sakurai A, Tonelli F & Brandi ML. Clinical practice guidelines for multiple endocrine neoplasia type 1 (MEN1). Journal of Clinical Endocrinology and Metabolism 2012 97 2990–3011. (doi:10.1210/jc.2012-1230)
2 Lemos MC & Thakker RV. Multiple endocrine neoplasia type 1 (MEN 1) analysis: of 1336 mutations reported in the first decade following identification of the gene. Human Mutation 2008 29 22–32. (doi:10.1002/humu.20605)
3 Concolino P, Costella A & Capoluongo E. Multiple endocrine neoplasia type 1 (MEN1): an update of 208 new germline variants reported in the last nine years. Cancer Genetics 2016 209 36–41. (doi:10.1016/j.cancergen.2015.12.002)
4 Hackeng WM, Brosens LA, Poruk KE, Neo M, Hosoda W, Poling JS, Rizzo A, Campbell-Thompson M, Atkinson MA, Konukiewitz B, et al. Aberrant Menin expression is an early event in pancreatic neuroendocrine tumorigenesis. Human Pathology 2016 56 93–100. (doi:10.1016/j.humpath.2016.06.006)
5 Thakker RV. Multiple endocrine neoplasia type 1 (MEN1) and type 4 (MEN4). Molecular and Cellular Endocrinology 2014 386 2–15. (doi:10.1016/j.mce.2013.08.002)
6 Modlin IM, Oberg K, Chung DC, Jensen RT, de Herder WW, Thakker RV, Caplin M, Delle Fave G, Kaltas GA, Krenning EP, et al. Gastroneuroendarocrine neuroendocrine tumours. Lancet Oncology 2009 9 61–72. (doi:10.1016/S1470-2045(07)70410-2)
7 Frilling A, Akerstrom G, Falconi M, Pavel M, Ramos J, Kidd M & Modlin IM. Neuroendocrine tumor disease: an evolving landscape. Endocrine-Related Cancer 2012 19 R163–185. (doi:10.1530/ERC-12-0024)
8 Yates CJ, Newey PJ & Thakker RV. Challenges and controversies in management of pancreatic neuroendocrine tumours in patients with MEN1. Lancet Diabetes and Endocrinology 2015 3 895–905. (doi:10.1016/S2213-8587(15)00043-1)
9 Jiao Y, Shi C, Edil BH, de Wilde RE, Klimstra DS, Maitra A, Schlicker RD, Tang LH, Wolfgang CL, Choti MA, et al. DAXX/ATRX, MEN1, and mdTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. Science 2011 331 1119–1203. (doi:10.1126/Science.1200609)
10 Matkar S, Thiel A & Hua X. Menin: a scaffold protein that controls gene expression and cell signalling. Trends in Biochemical Sciences 2013 38 394–402. (doi:10.1016/j.tibs.2013.05.005)
11 Huang J, Gurung B, Wan B, Matkar S, Veniaminova NA, Wan K, Merchant JL, Hua X & Lei M. The same pocket in menin binds both MLL and JUND but has opposite effects on transcription. Nature 2012 482 542–546. (doi:10.1038/nature10806)
12 Milne TA, Hughes CM, Lloyd R, Yang Z, Rozenblatt-Rosen O, Dou Y, Schnepp RW, Kranck E, Livolsi VA, Gibbs D, et al. Menin and MLL cooperatively regulate expression of cyclin-dependent kinase inhibitors. PNAS 2005 102 749–754. (doi:10.1073/pnas.0408836102)
13 Hussein N, Casse H, Fontaniere S, Morera AM, Asensi MJ, Bakeli S, Lu J, Coste L, Di Clemente N, Bertolino P, et al. Reconstituted expression of menin in Men1-deficient mouse Leydig tumour cells induces cell cycle arrest and apoptosis. European Journal of Cancer 2007 43 402–414. (doi:10.1016/j.ejca.2006.08.038)
14 Bertolino P, Tong WM, Galendo D, Wang ZQ & Zhang CX. Heterozygous Men1 mutant mice develop a range of endocrine tumours mimicking multiple endocrine neoplasia type 1. Molecular Endocrinology 2003 17 1880–1892. (doi:10.1210/me.2003-0154)
15 Crabtree JS, Schachler PC, Ward JM, Garrett-Real L, Emmert-Buck MR, Edgemon KA, Loran D, Libutti SK, Chandrasekharraapa SC, Marx SJ, et al. A mouse model of multiple endocrine neoplasia, type 1, develops multiple endocrine tumours. PNAS 2001 98 1118–1123. (doi:10.1073/pnas.98.3.1118)
MEN1 mouse model under temporal control

16 Harding B, Lemos MC, Reed AA, Walls GV, Jeyabalan J, Bowl MR, Tatesossian H, Sullivan N, Hough T, Hambardzhiev D, et al. Multiple endocrine neoplasia type 1 knockout mice develop parathyroid, pancreatic, pituitary and adrenal tumours with hypercalcaemia, hypophosphataemia and hypercorticoestrogenism. Endocrine-Related Cancer 2009 16 1313–1327. (doi:10.1677/ERC-09-0082)

17 Loffler KA, Biondi CA, Gartside M, Waring P, Stark M, Serewko-Auret MM, Muller HK, Hayward NK & Kay GF. Broad tumour spectrum in a mouse model of multiple endocrine neoplasia type 1. International Journal of Cancer 2007 120 259–267. (doi:10.1002/ijc.22288)

18 Wiedemann T & Pellegrata NS. Animal models of multiple endocrine neoplasia. Molecular and Cellular Endocrinology 2016 421 49–59. (doi:10.1016/j.mce.2015.07.004)

19 Bill R, Fagiani E, Zumsteg A, Antoniadi H, Johansson D, Haefliger S, Fagiani E, Zumsteg A, Johansson D, Haefliger S, Wiedemann T & Pellegata NS. Animal models of multiple endocrine neoplasia. Endocrinology 2016 157 153–161. (doi:10.1210/en.2015-1194)

20 Jiang X, Cao Y, Li F, Su Y, Li Y, Peng Y, Cheng Y, Zhang C, Wang W & Ning G. Targeting beta-catenin signaling for therapeutic intervention in MEN1-deficient pancreatic neuroendocrine tumours. Nature Communications 2014 5 6809. (doi:10.1038/ncomms6809)

21 Raymond E, Dahan L, Raoul JL, Jiang X, Bortolotto S, Lembhard-Bohas C, Valle J, Metzger D, Smith D, Tink A, et al. Sunstihibn malate for the treatment of pancreatic neuroendocrine tumours. New England Journal of Medicine 2011 364 501–513. (doi:10.1056/NEJMoa1003825)

22 Sennino B, Ishiguro-Onuma T, Wei Y, Naylor RM, Williamson CW, Bhagwandin V, Tabruyn SP, You WK, Chapman HA, Christofori G. Of mice and men: Molecular and Cellular Endocrinology type 1 in mouse models for basic and preclinical studies. Science 2004 303 156 5167–5179. (doi:10.1126/science.2010.009128)

23 Walls GV, Lemos MC, Javid M, Bazan-Peregrino M, Jeyabalan J, Reed AA, Harding B, Tyler DJ, Stuckey DJ, Fret S, et al. MEN1 gene replacement therapy reduces proliferation rates in a mouse model of pituitary adenomas. Cancer Research 2012 72 5060–5068. (doi:10.1158/0008-5472.CAN-12-1821)

24 Walls GV, Stevenson M, Soukup BS, Lines KE, Grossman AB, Schmid HA & Thakker RV. Paracoidet therapy of multiple endocrine neoplasia type 1-associated neuroendocrine tumours in female mice deleted for an men1 allele improves survival and reduces tumor progression. Endocrinology 2016 157 1719–1798. (doi:10.1210/en.2015-1965)

25 Feil R, Birod J, Mascrez B, LeMeur M, Metzger D & Chambon P. Ligand-activated site-specific recombination in mice. PNAS 1996 93 10887–10890. (doi:10.1073/pnas.93.20.10887)

26 Lines KE, Stevenson M & Thakker RV. Animal models of pituitary neoplasia. Molecular and Cellular Endocrinology 2016 421 68–81. (doi:10.1016/j.mce.2015.08.024)

27 Bertolino P, Tong WM, Herrera PL, Casse H, Zhang CX & Wang ZQ. Pancreatic beta-cell-specific ablation of the multiple endocrine neoplasia type 1 (MEN1) gene causes full penetrance of insulinoma development in mice. Cancer Research 2003 63 4836–4841.

28 Crabtree JS, Scacheri PC, Ward JM, McNally SR, Swain GP, Montagna C, Hager JH, Hanahan D, Edlund H, Magnuson MA, et al. Of mice and MEN1: insulinomas in a conditional mouse knockout. Molecular and Cellular Biology 2003 23 6075–6085. (doi:10.1128/MCB.23.17.6075-6085.2003)

29 Biondi CA, Gartside MG, Waring P, Loffler KA, Stark MS, Magnuson MA, Kay GF & Hayward NK. Conditional inactivation of the MEN1 gene leads to pancreatic and pituitary tumorigenesis but does not affect normal development of these tissues. Molecular and Cellular Biology 2004 24 3125–3131. (doi:10.1128/MCB.24.8.3125-3131.2004)

30 Li F, Su Y, Cheng Y, Jiang X, Peng Y, Li Y, Lu J, Gu Y, Zhang C, Cao Y, et al. Conditional deletion of Men1 in the pancreatic beta-cell leads to glucagon-expressing tumor development. Endocrinology 2015 156 48–57. (doi:10.1210/en.2014-1433)

31 Snchez RW, Chen YX, Wang H, Cash T, Silva A, Diehl JA, Brown F & Hua M. Mutation of tumor suppressor gene Men1 acutely enhances proliferation of pancreatic islet cells. Cancer Research 2006 66 5707–5715. (doi:10.1158/0008-5472.CAN-05-4518)

32 Dor Y, Brown J, Martinez OI & Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004 429 41–46. (doi:10.1038/nature02520)

33 Libutti SK, Cranberry JS, Lorang DB, Burns AL, Mazzanti C, Hewitt SM, O’Connor S, Ward JM, Emmert-Buck MR, Remaley A, et al. Parathyroid gland-specific deletion of the mouse Men1 gene results in parathyroid neoplasia and hypercalcemic hyperparathyroidism. Cancer Research 2003 63 8022–8028. (doi:10.1089/crneo.2010.1311)

34 Stechman MJ, Ahmad BN, Loh NY, Reed AA, Stewart M, Wells S, Hough T, Bentley L, Cox RD, Brown SD, et al. Establishing normal plasma and 24-hour urinary biochemistry ranges in C3H, BALB/c and C57BL/6 mice inoculated with melanoma tumor cells. Laboratory Animals 2010 44 218–225. (doi:10.1258/lab.2010.009128)

35 Walls GV, Reed AA, Jeyabalan J, Javid M, Hill NR, Harding B & Thakker RV. Proliferation rates of multiple endocrine neoplasia type 1 (MEN1)-associated tumors. Endocrinology 2012 153 5167–5179. (doi:10.1210/en.2012-1675)

36 Cheng Y, Su T, Shan A, Jiang X, Ma Q, Wang W, Ning G & Cao Y. Generation and characterization of transgenic mice expressing Men1 promoter for pancreatic beta-cell-specific gene overexpression and knockout. Endocrinology 2015 156 2724–2731. (doi:10.1210/en.2015-1104)

37 Gannon M, Shiotia C, Potic C, Wright CV & Magnuson M. Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. Genesis 2005 42 139–142. (doi:10.1002/geo.1526-968X(20002)26:2<139::AID-GENE12>3.0.CO;2-7)

38 Burden N, Chapman K, Sewell F & Robinson V. Pioneering better science through the 3Rs: an introduction to the national centre for the replacement, refinement, and reduction of animals in research (NCR3Rs). Journal of the American Association for Laboratory Animal Science 2015 54 198–208.

39 Agarwal SK. Exploring the tumors of multiple endocrine neoplasia type 1 in mouse models for basic and preclinical studies. International Journal of Endocrine Oncology 2014 1 153–161. (doi:10.2217/ijoe.14.16)

40 Puri S, Follas AE & Hrebek M. Plasticity and dedifferentiation within endocrine-related cancer. International Journal of Cancer.1.01294)

41 Lejonklu M, Barbu A, Stalberg P & Skogseid B. Accelerated proliferation and differential global gene expression in pancreatic islets of five-week-old heterozygous Men1 mice: Men1 is a haploinsufficient suppressor. Endocrinology 2012 153 2588–2598. (doi:10.1210/en.2011-1924)

42 Fontaniere S, Tost J, Wierinckx A, Lachuer J, Lu J, Hussein N, Busato F, Gut I, Wang ZQ & Zhang CX. Gene expression profiling in insulinsomas of Men1 beta-cell mutant mice reveals early genetic and epigenetic events involved in pancreatic beta-cell tumorigenesis. Endocrine-Related Cancer 2006 13 1223–1236. (doi:10.1677/erc.1.01294)