Conditional Deletion of *Men1* in the Pancreatic \(\beta\)-Cell Leads to Glucagon-Expressing Tumor Development

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The tumor suppressor menin is recognized as a key regulator of \(\beta\)-cell proliferation. To induce tumorigenesis within the pancreatic \(\beta\)-cells, floxed alleles of *Men1* were selectively ablated using Cre-recombinase driven by the insulin promoter. Despite the \(\beta\)-cell specificity of the RipCre, glucagon-expressing tumors as well as insulinomas developed in old mutant mice. These glucagon-expressing tumor cells were menin deficient and expressed the mature \(\alpha\)-cell-specific transcription factors Brain-specific homeobox POU domain protein 4 (Brn4) and \(\nu\)-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB). Moreover, the inactivation of \(\beta\)-cell-specific transcription factors was observed in mutant \(\beta\)-cells. Our work shows that *Men1* ablation in the pancreatic \(\beta\)-cells leads to the inactivation of specific transcription factors, resulting in glucagon-expressing tumor development, which sheds light on the mechanisms of islet tumorigenesis.

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Various endocrine cell types can give rise to pancreatic neuroendocrine tumors (PNETs), which are broadly classified into functional (hormone producing) and non-functional tumors (1). In humans, glucagonomas have been observed to arise considerably less frequently than insulinomas (2). Glucagonomas appear to primarily occur sporadically but are infrequently found in association with multiple endocrine type 1 neoplasias (MEN1s) and von Hippel-Lindau syndromes, and glucagonomas can be multicentric in such cases (1).

The mechanisms responsible for the development of glucagonomas are not well understood. A recent genetic study demonstrated that 44.1% of sporadic PNETs harbor inactivating somatic mutations in the *Men1* gene (3). Thus, aberrant menin expression in \(\alpha\)-cells per se has frequently been associated with the development of sporadic and MEN1-related glucagonomas. Additionally, a patient with an inactivating glucagon receptor (GCGR) mutation has been found to exhibit hyperglucagonemia and pancreatic \(\alpha\)-cell hyperplasia, which suggests that the GCGR signaling pathway likely contributes to the development of glucagonomas (4), and this suggestion has been verified in a study of a mouse model of GCGR deficiency (5). Mutations of p53 have been observed in human PNETs (6). A recent study strongly supported more extensive involvements of the p53 and Rb pathways in the development of glucagonomas (7). Interestingly, the findings from Lu et al (8) highlight cell transdifferentiation as a novel mechanism that is involved in islet tumor development. The observations of these authors indicate that \(\alpha\)-to \(\beta\)-cell
reprogramming is a common phenomenon in MEN1-related insulinoma development. Several lines of evidence suggest that the plasticity and maturity of islet cells can be altered when the quantitative balance is broken (8, 9) and that the reciprocal transdifferentiation of β- and α-cells might be regulated by context-specific transcription factors, such as neurogenin-3 (Ngn3) (10, 11), NK2 homeobox 2 (Nkx2.2) (12), and paired box transcription factor (Pax)-4 (13, 14). Thus, the mechanisms underlying glucagonoma development remain elusive. Exploring these mechanisms will help to optimize therapeutic intervention strategies.

Clinically it has been reported that insulinomas can transform into glucagonoma syndrome (15–17). Moreover, the coexistence of a glucagonoma with a recurrent insulinoma has been reported in a patient with MEN1 (18). These clinical findings support the hypothesis that delayed glucagonomas might originate from hyperplastic β-cells.

Different β-cell-specific Men1 knockout mice models have been generated to recapitulate the pathomorphological features of insulinomas. However, in addition to islet tumors, pituitary adenomas have also been detected in these models (19, 20). In our mouse model, no tumors were detected outside of the pancreas, which is likely due to the improved RipCre mouse line. We used this mouse model to study the pathology and the histogenesis of MEN1-associated PNETs (21–24). Serendipitously, in addition to insulinomas, glucagonomas and mixed glucagon- and insulin-expressing islet tumors developed in these Men1 mutant mice when they reached advanced ages. Inactivations of specific β-cell transcription factors were also observed in the mutant β-cells. Therefore, our study established a novel mechanism that is associated with glucagonoma histogenesis and might contribute to glucagonoma diagnosis and therapy.

Materials and Methods

Animals

Men1<sup>fl</sup>x<sup>fl</sup> mice (21–24) were bred with mice expressing Cre recombinase driven by the rat insulin promoter (RipCre) to generate the conditional Men1 knockout mice (Men1<sup>fl</sup>x<sup>fl</sup>-RipCre<sup>Cre</sup>). Men1<sup>fl</sup>x<sup>fl</sup>-RipCre<sup>Cre</sup> mice were used as the control. Heterozygous (Men1<sup>fl</sup>x<sup>fl</sup>-RipCre<sup>Cre</sup>) mice were backcrossed with C57BL/6J mice five times and then crossed to generate homozygous mice. The C57BL/6J mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. All of the mice were housed in pathogen-free facilities with a 12-hour light, 12-hour dark cycle and had free access to water and food. The R26R mice used in this study have been previously described (8). Only male mice were used for fasting blood glucose and serum glucagon test. Both male and female mice were used for histo-

logical and immunostaining analysis. All of the animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Metabolic measurements

Serum glucagon measurements were analyzed from collected orbit blood with the use of a mouse ELISA kit (Merkodia). Animals were deprived of food 6 hours before the blood collection, and duplicate measurements were repeated twice for each sample. Glucose concentrations were measured from tail blood using One-Touch Ultra glucometers (LifeScan).

Histological and immunostaining analyses

Pancreata were harvested at different ages and fixed in 4% buffered formaldehyde. The immunohistological analyses were performed on paraffin serial sections, as described previously (21–24). For the antibodies used for the immunochemistry and immunofluorescence assays, see Supplemental Materials and Methods. The images were acquired using a Zeiss confocal microscope or an Olympus system.

Isolation of mouse pancreatic islets and microdissection

Pancreatic islets were isolated from mice at 2–15 months of age as previously described (21). Briefly, the pancreases were digested with collagenase and dissociated vigorously by mechanical pipetting. The islets were handpicked from dark-field dishes under a dissecting microscope and pooled for further analysis. Frozen pancreas tissue sections were stained with hematoxylin and eosin (HE) first, and then the adjacent section was immunostained by antiguclagon antibody to confirm the identity of the tumor. If the tumor was judged as a glucagonoma, manual microdissection was performed under a microscope to sample the relevant area on the serial section slides with a fine needle.

Genotyping and PCR analysis

Genotyping analysis was performed as previously described (21–24). The DNA was extracted and amplified using the following three primers: I2f0, 5’-CTACCTCTTCTCATGTCTG; E3f1, 5’-GGATTCTGCTCCCCAGGC; and E3r1, 5’-CACC TCCATCTTACGGTGCG. For more information, see Supplemental Materials and Methods.

Quantitative PCR analysis

The total RNA extraction was performed on 6- to 12-month-old hand-picked islets using an RNeasy kit (QIAGEN) according to the manufacturer’s instructions. Quantitative real-time PCRs were performed as previously described (22). Primer sequences are provided in Supplemental Materials and Methods.

Statistical analysis

All of the results are reported as the means ± SEM. Each variable was analyzed with unpaired Student’s t test. For all of the analyses, values of P < .05 were considered significant. All of the analyses were performed using the GraphPad Prism software (GraphPad Software Inc).
Results

*Men1* is conditionally ablated in pancreatic β-cells

To ablate the *Men1* gene in pancreatic β-cells, *Men1*<sup>ff</sup>-mice were crossed with RipCre<sup>+</sup> mice expressing Cre recombinase driven by the insulin promoter (25). Using this Cre/loxP-based approach, exon 3 of the *Men1* gene was ablated (Figure 1A). The cell-specific *Men1* ablation was then examined in *Men1*<sup>ff</sup>-RipCre<sup>+</sup> mice. As expected, the excision of the floxed *Men1* allele was detected by PCR analysis in the pancreatic islets isolated from 8-week-old *Men1*<sup>ff</sup>-RipCre<sup>+</sup> mice but not in other tissues (Figure 1B). *Men1* inactivation was further confirmed by immunofluorescence. Menin-negative β-cells could be observed as early as embryonic day (E) 17.5 in the embryos of *Men1*<sup>ff</sup>-RipCre<sup>+</sup> mice (Figure 1C). Menin expression was lost in 90% of the insulin-expressing cells in the pancreas of *Men1*<sup>ff</sup>-RipCre<sup>+</sup> mice at 8 weeks of age (Figure 1D). Conversely, the glucagon-expressing cells remained menin positive at this age (Figure 1D). We also checked the β-cell specificity of RipCre in the endocrine pancreas using RipCre<sup>-</sup>-R26R mice. β-Galactosidase expression was exclu-
sively detected in the insulin-producing cells in the pancreata of RipCre<sup>+</sup>-R26R mice at 8 weeks of age (Figure 1E). No labeling was found in the glucagon-producing cells (Figure 1E). Altogether these results confirm the β-cell-specific Men1 disruption in the mutant mice.

Men1 ablation promotes β-cell proliferation and islet hyperplasia. Mcm2 and Ki67 are two representative markers of β-cell proliferation. Immunofluorescence staining showed that the numbers of Mcm2- and Ki67-positive β-cells were significantly increased in the islets of

![Figure 2](https://example.com/figure2)

**Figure 2.** Ablation of the Men1 gene in β-cells leads to glucagonoma development. A and B, Fasting blood glucose and serum glucagon levels monitored in 15-month-old Men1<sup>f/f</sup>-RipCre<sup>+</sup> mice. The circle indicates that higher blood glucose levels (A) are observed in two Men1<sup>f/f</sup>-RipCre<sup>+</sup> mice and their fasting serum glucagon levels (B) are also significantly increased. C, Representative micrographs of HE and glucagon staining showing that glucagon-expressing tumors are detected in the Men1<sup>f/f</sup>-RipCre<sup>+</sup> mice with the higher blood glucose levels. D, Transcript factors, MafB, Brn4, Pdx-1, and Glut2, are detected in glucagon-expressing tumor cells. Gcg, glucagon. Scale bar, 50 μm.
the \textit{Men1}^{flfl}\textunderscore RipCre}^{+} compared with the control mice (Supplemental Figure 1).

**\textit{\beta}-Cell-specific \textit{Men1} ablation leads to glucagonoma development**

At 15 months of age, consistent with hypoglycemia, hyperplastic islets and insulinomas were clearly observed in \textit{Men1}-ablated mice. Surprisingly, the monitoring of the fasting blood glucose levels revealed higher concentrations in a small proportion of \textit{Men1}-ablated mice than in the others (Figure 2A). Moreover, the fasting serum glucagon concentrations were also significantly increased in these mice (Figure 2B). The necropsy of these mice detected islet tumors that nearly exclusively expressed glucagon (Figure 2C). The key transcription factors that appear to define the fully differentiated \(\alpha\)-cell phenotype are v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MafB) and Brain-specific homeobox POU domain protein 4 (Brn4) \((26, 27)\). As expected, MafB and Brn4 were found to be expressed in these glucagon-positive islet tumors, but pancreatic duodenal homeobox-1 (Pdx-1) and glucose transporter-2 (Glut2), which were regarded as functional markers of mature \(\beta\)-cells \((28)\), were not found to be expressed (Figure 2D). Thus, these glucagon-expressing tumors were glucagonomas because the cells in them exhibited the differentiated \(\alpha\)-cell phenotype. HE staining showed that additional interstitial tissues could be observed in the glucagonomas compared with the insulinomas, and the nuclei were arranged more compactly in the glucagonomas (Figure 3A) and were more easily observed with Hoechst staining (Figure 3B). In fact, according to these morphological differences, glucagonoma could be preliminarily and conveniently discriminated from insulinoma in \textit{Men1}-ablated mice. The numbers of Mcm2- and Ki67-positive cells were significantly increased in glucagonomas and insulinomas (Figure 3C), which suggests that the cells in glucagonomas are also significantly proliferative.

**Figure 3.** Differences and resemblances between glucagonoma and insulinoma in \textit{Men1}^{flfl}\textunderscore RipCre}^{+} mice. A and B, Morphological differences (A) between glucagonoma and insulinoma and the difference in the nuclear arrangement between \(\beta\)- and \(\alpha\)-cells in mixed tumor (B) are demonstrated by HE and immunofluorescence staining. The dashed line circles the nuclei of \(\alpha\)-cells in mixed tumors (B, right panel). C, Immunofluorescence analysis of Ki67 and Mcm2 expression in glucagonoma and insulinoma. D, Proportions of the glucagonomas observed in \textit{Men1}^{flfl}\textunderscore RipCre}^{+} and control mice at 12, 15, and 18 months of age. Gcg, glucagon. Scale bar, 50 \(\mu\)m.
Men1\textsuperscript{f/f}-RipCre\textsuperscript{+} mice were monitored over an 18-month period and necropsied at different ages. Before 12 months of age, no glucagonomas were detected. At 15 months of age, the proportion of islet tumors exclusively expressing glucagon was 15\% (n = 20), and this proportion increased to 35\% (n = 20) at 18 months of age (Figure 3D). No islet tumors were observed in the control mice.

At 14 months of age, islets with hyperplastic \(\alpha\)-cells (IHA) and mixed tumors expressing both insulin and glucagon could be observed in Men1\textsuperscript{f/f}-RipCre\textsuperscript{+} mice (Figure 4), suggesting that glucagonoma development is an accumulative and consecutive pathological process. Glucagon-expressing cells in IHA and mixed tumors also expressed the transcription factors MafB and Brn4 (Figure 4), which define the fully differentiated \(\alpha\)-cell phenotype.

Occurrence of glucagonomas is not due to adaptive \(\alpha\)-cell proliferation

\(\alpha\)-Cells produce glucagon to maintain glucose homeostasis in response to hypoglycemia by stimulating hepatic glucose production. It has been hypothesized that the development of glucagonomas in Men1\textsuperscript{f/f}-RipCre\textsuperscript{+} mice could be due to adaptive \(\alpha\)-cell proliferation that was triggered by the chronic hypoglycemia that occurs in old mutant mice. Almost all of the glucagon-expressing cells in IHA and mixed tumors exhibited a loss in menin expression (Figure 5A). However, islets with nonhyperplastic \(\alpha\)-cells (INA) juxtaposed with IHA could be observed in the same Men1\textsuperscript{f/f}-RipCre\textsuperscript{+} pancreas, and menin could be detected in the \(\alpha\) cells of INA (Figure 5B). Moreover, the numbers of Ki67-positive \(\alpha\)-cells in IHA were significantly increased compared with those in INA (Figure 5C), suggesting that glucagonoma development is not due to proliferation by adaptive normal \(\alpha\)-cells but to the proliferation of menin-deficient \(\alpha\)-cells. Noticeably, cells coexpressing glucagon and insulin could also be observed in some IHA (Figure 5D), and this pattern was never detected in normal control islets.

We also analyzed the status of the Men1 gene in the glucagonomas. The genotyping of microdissected glucagonomas showed that the glucagonoma cells, similarly to the insulinoma cells, contained the deleted Men1 allele, whereas the exocrine pancreatic cells uniquely displayed the intact floxed Men1 allele (Figure 5E).

Inactivation of specific \(\beta\)-cell transcription factors is detected in Men1-ablated islets

Some transcription factors, such as Nkx6.1, Nkx2.2, Pax4, neurogenic differentiation-1 (NeuroD1), Isl-1, Pax6, Forkhead box-a2 (Foxa2), and \(\nu\)-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (MafA), have been identified to play important roles in \(\beta\)-cell differentiation, maturation, and function maintenance (29). The mRNA levels of Nkx6.1, NeuroD1, Isl-1, and MafA were significantly lower in the islets and solid tumors of Men1-ablated mice compared with control mice, whereas the level of Nkx2.2 was significantly lower only in the islets, and the levels of Pax6, Foxa2 were significantly lower only in the solid tumors of Men1-ablated mice (Figure 6A). Moreover, the Nkx6.1, Nkx2.2, NeuroD1, and Pax4 protein levels were significantly reduced in the islets of Men1-ablated mice (Figure 6B).

MafA is a transcription factor that is tightly restricted to the \(\beta\)-cell nucleus in adult islets and is necessary for

\begin{figure}[h]
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\caption{Representative images of IHA and mixed tumors observed in Men1\textsuperscript{f/f}-RipCre\textsuperscript{+} mice at 14 months of age. MafB and Brn4 are detected in IHA and mixed tumor cells. Gcg, glucagon.}
\end{figure}
Figure 5. Glucagonoma cells originate from β-cells in Men1<sup>f/f</sup>-RipCre<sup>+</sup> mice. A, Loss of menin expression detected in IHA and mixed tumors. B, Menin expression in IHA and INA in the same Men1<sup>f/f</sup>-RipCre<sup>+</sup> pancreas. Only α-cells in IHA lost their menin expression. C, Ki67 expression detected in IHA and INA in the same Men1<sup>f/f</sup>-RipCre<sup>+</sup> pancreas. The quantification of the percentage of α-cells containing Ki67 is shown. D, Cells coexpressing glucagon and insulin observed in IHA. E, Deletion of the Men1 allele (Δ) detected by PCR in both microdissected β- and α-cell tumors. f, floxed Men1 allele. F, MafB expression detected in β-cells from hyperplastic islets. The inset shows MafB<sup>+</sup> insulin<sup>+</sup> β-cells in Men1<sup>f/f</sup>-RipCre<sup>+</sup> mice. Gcg, glucagon. Scale bar, 25 μm.
optimal insulin gene expression (30). The MafA protein levels in the islets of Men1-ablated mice and control mice at 2 months of age were similar. However, at 4 months of age, the level of this transcription factor was significantly reduced in the islets of Men1-ablated mice compared with control mice. At 12 months of age, the MafA protein level in the Men1-ablated mice was further decreased and only 45.7% of that found in the control mice (Figure 6, C and D). Thus, these results suggest that the gradual decrease in the expression of specific transcription factors affects β-cell maturation and plasticity.

It is worth noting that the mRNA level of snail family zinc finger 2 (Snail2) was significantly increased in the islets and solid tumors of Men1-ablated mice (Figure 6A). Snail2 is the direct target of Ngn3 and contributes to the delamination of differentiating endocrine cells during pancreas development (31), which can be regarded as an endocrine progenitor marker (29).

**Discussion**

In this study, we demonstrated that β-cell-specific Men1 ablation can decrease the expression of specific transcription factors and result in glucagon-expressing islet tumor development. It has been reported that menin can regulate α-cell plasticity, and menin-deficient α-cells can transdifferentiate into insulin-expressing cells, leading to insuli-
oma development (8), establishing α-cell reprogramming to the β-cell fate as a novel mechanism underlying islet tumorigenesis. A similar mechanism may exist in the tumor progression process in our mouse model. Multiple lines of evidence support that the menin-deficient α-cells might originate from β-cells, which are the following: 1) the β-cell specificity of RipCre; 2) the same genetic modification of the Men1 gene in the glucagonoma cells as well as in the insulinoma cells; 3) the proposed intermediates in the transdifferentiation process, such as insulin⁺/glucagon⁺ cells, MafB⁺/insulin⁺ cells, and IHA; and 4) inactivation of specific transcription factors in β-cells.

Based on the specificity of the Cre/loxP approach, we believe that the loss of menin expression in α-cells was not ascribed to the leakage of RipCre-mediated recombination. If the leakage existed, the menin inactivation in α-cells, as in β-cells, should be an early event. In fact, persistent menin expression in α-cells was detected both at the embryo stage and after birth. Moreover, IHA and INA coexisted in the same pancreas of Men1⁺⁻/RipCre⁺ mice at 14 months of age and older. In view of the spatiotemporal expression feature of menin in α-cells, the menin inactivation in α-cells was ascribed to transdifferentiation from β-cells rather than direct ablation by RipCre.

The plasticity of fully mature cells in the pancreas has been an attractive and controversial research area. Studies accomplished in mice programmed with cell lineage-tracking markers in vivo have revealed the remarkable plasticity of islet endocrine cells. α-Cells transdifferentiate into β-cells and vice versa (8, 9, 32). In Men1⁺⁻/RipCre⁺ mice, hypoglycemia appears at a very young age. Intriguingly, despite chronic hypoglycemia, normal menin-expressing β-cells rather than direct ablation by RipCre.

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In Men1⁺⁻/RipCre⁺ mice, the glucagon-expressing cells express MafB and Brn4, which are regarded as mature α-cell-specific markers. During development, MafB is essentially absent from mature adult β-cells, and its expression is restricted to α-cells in the adult pancreas (29, 33, 34). Specifically, the heterozygous expression of MafB can be detected in insulin-expressing cells. However, further studies are required to determine whether these MafB⁺/insulin⁺ cells are the direct progenitors of transdifferentiated α-cells.

Our results indicate that a specific subset of transcription factors, which define the full differentiation phenotype of β-cells, is attenuated in the mutated mice. These transcription factors, including Nkx6.1 (35), Nkx2.2 (36, 37), Pax4 (14), NeuroD1 (38), and MafA (30, 39, 40), are essential not only for the differentiation of β-cells but also to glucose sensing, insulin synthesis, and insulin secretion. Thus, our findings indicate that a loss in islet-enriched transcription factor activity affects β-cell differentiation, maturation, and function. Moreover, the inactivation of Nkx2.2 and Pax4 may promote the transdifferentiation. In Men1⁺⁻/RipCre⁺ mice, and the expression of Nkx2.2 and Pax4 was significantly decreased. No active Ngn3 expression, which is a specific marker in endocrine progenitor, was detected in mature Men1⁺⁻/RipCre⁺ mice. Further study is required to determine whether the transdifferentiated α-cells are direct descendants of β-cells or whether they arise independently from an undifferentiated endocrine progenitor that originated from dedifferentiated β-cells.

In conclusion, the current study shows that menin ablation can promote β-cell dedifferentiation and might enhance β-cell plasticity. Therefore, our results provide novel insights into the genetic events that regulate plasticity in mature β-cells and shed light on islet tumorigenesis.

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