Tumour suppressor menin is essential for development of the pancreatic endocrine cells

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Abstract

Mutations of the multiple endocrine neoplasia type 1 (*MEN1*) gene predispose patients to MEN1 that affects mainly endocrine tissues, suggesting important physiological functions of the gene in adult endocrine cells. Homozygous disruption of *Men1* in mice causes embryonic lethality, whereas the eventual involvement of the gene in embryonic development of the endocrine cells remains unknown. Here, we show that homozygous *Men1* knockout mice demonstrate a reduced number of glucagon-positive cells in the E12.5 pancreatic bud associated with apoptosis, whereas the exocrine pancreas development in these mice is not affected. Our data suggest that menin is involved in the survival of the early pancreatic endocrine cells during the first

developmental transition. Furthermore, chimerism assay revealed that menin has an autonomous and specific effect on the development of islet cells. In addition, using pancreatic bud culture mimicking the differentiation of α - and β -cells during the second transition, we show that loss of menin leads to the failure of endocrine cell development, altered pancreatic structure and a markedly decreased number of cells expressing neurogenin 3, indicating that menin is also required at this stage of the endocrine pancreas development. Taken together, our results suggest that menin plays an indispensable role in the development of the pancreatic endocrine cells.

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Introduction

Mutations of the multiple endocrine neoplasia type 1 (*MEN1*) gene are found in the majority of patients with MEN1, predisposing them to the occurrence of multiple endocrine tumours of the parathyroids, endocrine pancreas and anterior pituitary, as well as, though less frequently, of foregut carcinoids, adrenal cortical tumours and follicular thyroid tumours (Online Mendelian Inheritance in Man no. 131100). While hyperparathyroidism is the most common clinical manifestation of the disease, pancreatic islet tumours, including those of α -, β - and pancreatic polypeptide (PP) cells, occur in 30–80% of MEN1 patients and account for the major cause of death in MEN1 patients due to malignancy (Thakker 1998). The loss of heterozygosity observed in MEN1 tumours (Larsson *et al.* 1988) supports the hypothesis that the *MEN1* gene acts as a tumour suppressor in affected cells.

The protein product of the *MEN1* gene, menin, has been shown to interact with a substantial number of well-known transcriptional factors and co-factors, including JUND

(Agarwal *et al.* 1999), SMAD1, 3 and 5 (Kaji *et al.* 2001, Sowa *et al.* 2004), RELA, NF κ B2 and NF κ B1 of NF κ B (Heppner *et al.* 2001), SIN3A (Kim *et al.* 2003), MLL1 and 2 (Hughes *et al.* 2004, Yokoyama *et al.* 2004, Milne *et al.* 2005), suggesting that menin is involved in transcriptional regulation of gene expression. Indeed, several genes have been identified as being regulated by menin, such as telomerase (Lin & Elledge 2003) and cyclin-dependent kinase inhibitors CDKN2C and CDKN1B (Karnik *et al.* 2007), as well as several hormones including insulin (Sayo *et al.* 2002), prolactin (Namiyama *et al.* 2002) and parathyroid hormone (Sowa *et al.* 2004). However, little is known about the *in vivo* physiological function of menin, and the *in vivo* importance of the above-mentioned interactions between menin and its protein partners, especially in endocrine cells that are affected in MEN1 disease. It should be noted that Kaji *et al.* (2001) has shown that menin is required for TGF β 1-induced *ex vivo* growth inhibition of anterior pituitary tumour cells and a similar observation has been made by Sowa *et al.* (2004) in primary parathyroid cells. By disrupting *Men1* in mice, we have previously shown that menin was

required for the development of several organs and tissues, such as neural tube and liver (Bertolino *et al.* 2003a). Consequently this elicits the question about the possible involvement of menin in the development of the pancreatic endocrine cells. Intriguingly, β -cell specific *Men1* mutant mice showed normal islet structures at an early age (Bertolino *et al.* 2003c, Crabtree *et al.* 2003, Biondi *et al.* 2004). However, one can hardly exclude the possibility that this may be due to partial Cre-mediated gene disruptions among targeted cells during the embryonic stage. Alternatively, menin may play its role before Rip-Cre recombinase (insulin) expression, similar to the genes whose function in the pancreas development is needed only during a narrow period, as in the case of β -catenin (Heiser *et al.* 2006).

Substantial advances in the study of development of the pancreatic endocrine cells have been achieved in recent years. The current available data support the notion that sequential activation or repression (Edlund 2002, Wilson *et al.* 2003) of transcriptional factors and co-factors may play a central role in the control of pancreas development. Indeed, the endodermal region committed to pancreas development first expresses pancreatic-duodenal homeobox 1 (PDX1), detected starting from E8.5 in the mouse embryos in early progenitors and restricted to the β -cells at the late developmental stage (Guz *et al.* 1995). Targeted disruption of the *Pdx1* gene in mice as well as homozygous mutation in human causes pancreas agenesis (Ahlgren *et al.* 1996, Stoffers *et al.* 1997), indicating its essential function in the morphogenesis and differentiation of the pancreas. The basic helix-loop-helix transcription factor neurogenin 3 (NEUROG3) is expressed transiently in the pancreatic endocrine progenitors (Apelqvist *et al.* 1999). *Neurog3*-deficient mice lack all pancreatic endocrine cell types (Gradwohl *et al.* 2000), indicating that it is required for differentiation of these cells. Further identification of the factors or co-factors involved in endocrine cell development would help to better understand the mechanisms implicated in this procedure. Considering the advance in the development of the pancreatic endocrine cells and available approaches allowing overcoming the lethality of *Men1* null embryos, we decided to determine the possible involvement of menin in the development of the pancreatic endocrine cells. In the current study, we have combined different genetic approaches to investigate the role of menin in pancreas development of the mouse. Our data provide compelling evidence showing that menin is essential for the development of the pancreatic endocrine cells and its absence results in the deficient formation of NEUROG3-positive cells, a key factor in this process.

Materials and Methods

Men1 knockout mice and generation of chimeric mice

Mice carrying the targeted *Men1* allele (*Men1*^{T/T}) or the deleted *Men1* allele (*Men1*^{Δ/Δ}) were used in the present study.

Both genotypes are null mutation in mice as described previously (Bertolino *et al.* 2003a). All animal experiments were conducted in accordance with the standards of human animal care and were approved by IARC's Animal Care and Use Committee.

For establishing *Men1* null ES cell lines, *Men1*^{+/T} mice were inter-crossed. Blastocysts were flushed from the uteri of pregnant female mice at day 3.5 *post coitum* and cultured individually on a murine fibroblastic cell layer for 5 days, in Dulbecco's modified Eagle's medium (15% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM β -mercaptoethanol and 10 ng/ml murine leukaemia inhibitory factor (Chemicon International, Temecula, CA, USA). The blastocysts were then dissociated with trypsin/EDTA, and put into culture for 7 days on a murine fibroblastic cell layer. Finally, the ES cells were amplified and karyotyped. To generate chimeric mice, two clones each of *Men1* wild-type and *Men1*^{T/T} ES cells were microinjected into blastocysts of C57BL/6 mice and transferred into pseudo-pregnant females.

Pancreas bud culture

The live embryos were harvested on E12.5. The pancreatic primordium was dissected and cultured on a filter (Millipore, Guyancourt, France) with RPMI 1640 medium (Invitrogen) containing penicillin (100 U/ml), streptomycin (100 µg/ml), HEPES (10 mmol/l), L-glutamine (2 mmol/l), non-essential amino acids (1×, Gibco), and 10% heat-inactivated serum (Hyclone, Logan, UT, USA) was added. Cultures were maintained at 37 °C in humidified 95% air, 5% CO₂.

Mouse islet isolation

Pancreatic islets were isolated from mice according to the protocol previously described (Bertolino *et al.* 2003c). Briefly, 2 ml of 1 mg/ml collagenase (SERVA, Heidelberg, Germany) in Hank's buffered saline solution was injected into the pancreas through the bile duct. Pancreases were then removed and incubated for 20 min at 37 °C and dissociated by mechanical pipetting. Islets were hand-picked from dark-field dishes under a dissecting microscopy.

Tissue collection and fixation

Tissues of chimeric mice were collected between 3 and 4 weeks after birth. Embryos were collected at different embryonic stages between E11.5 and E18.5. These tissues were fixed in 4% buffered formaldehyde and embedded in paraffin. The pancreatic rudiments were fixed at the end of the culture period in 4% buffered formaldehyde, pre-embedded in agarose (4% of type VII low gelling temperature agarose (Sigma)), followed by embedding in paraffin.

Histological, immunohistochemical and immunofluorescence analyses

For embryos and the pancreas of the chimeras, 3 µm sections were used either for staining with haematoxylin and eosin for histological analyses or for immunohistochemistry (IHC) and immunofluorescence (IF) staining as described previously (Bertolino *et al.* 2003b). Primary antibodies used were goat anti-menin C19 (1:500, Santa Cruz, CA, USA), rabbit anti-PDX1 (1:10 000 (Duvillie *et al.* 2003)) and mouse monoclonal anti-NEUROG3 (clone F25A1B3, 1:9000, DSHB). When using mouse antibodies as primary antibodies, Vector M.O.M. immunodetection kit was used (Vector Lab, Burlingame, CA, USA). For 5-bromodeoxyuridine (BrdU) analysis, bud cultures were incubated for 1 h with 10 µM BrdU (Sigma) before tissue fixation, then BrdU incorporation was evaluated by IHC using anti-BrdU antibody (Clone BU33 – Sigma). Primary antibodies used for IF experiments were guinea pig anti-insulin (1:500, Dako, Capintaria, CA, USA), rabbit anti-glucagon (1:600, NovoCastra, Milton Keynes, UK), anti-mucin-1 (1:200, Neomarkers, Fremont, CA, USA) and goat anti-menin (C19, 1:500).

For cultured pancreatic rudiments, 4 µm sections were processed for IF staining as previously described (Bhushan *et al.* 2001). The following antibodies were used: mouse anti-human insulin (1/2000, Sigma), rabbit anti-glucagon (1:1000, Diasorin, Stillwater, MN, USA), and rabbit anti-carboxypeptidase A (CPA1; 1:600, Biogenesis, Poole, UK). Quantification of the insulin- and glucagon-positive cells was performed with an IPLab 3.7 Eval software.

Apoptosis was examined on tissue sections by TUNEL staining using the 'in situ cell death detection' kit (Roche) and counterstained with DAPI (Vector Lab). Double TUNEL and glucagons analysis was done by combining the above-mentioned corresponding protocols.

In situ hybridization

For *in situ* hybridization, embryo tissues were fixed at 4 °C in 4% paraformaldehyde in PBS, cryoprotected in 15% sucrose–7.5% gelatin in PBS and frozen at –50 °C in isopentane. Cryosections (14 µm in thickness) were prepared. The *Neurog3* probe (726 bp) was prepared as previously described (Ravassard *et al.* 1997). Plasmids were linearized and used as templates for synthesizing sense or antisense riboprobes using T7 or SP6 RNA polymerase (Roche), in the presence of digoxigenin-UTP (Roche). *In situ* hybridization was performed as described previously (Duvillie *et al.* 2006), and colorimetric revelation was performed with 5-bromo-4-chloro-3-indolyl phosphate (Promega) and nitroblue tetrazolium (Roche), to obtain a blue precipitate. Photographs were taken using a Hamamatsu C5810 cooled 3CCD camera. No signal was obtained when a sense riboprobe was used.

PCR and quantitative RT-PCR

For PCR analysis of chimerism, genomic DNA was isolated from chimeric tissues at age of 3 weeks. The following

primers were used: NEO1-FW: 5'-GATGGATTGCACG-CAGGTTC-3'; NEO1-RV: 5'-CAGGTCGGTCTTGA-CAAAAAG-3'; B2-FW: 5'-CACCGGAGAATGGGAAG-CCGAA-3'; B2-RV: 5'-TCCACACAGATGGAGCGTC-CAG-3'.

For RT-PCR analysis, total RNA was prepared from E12.5 wild-type and *Men1*^{Δ/Δ} pancreas buds using Tri-Reagent (Sigma) according to the protocol provided by the manufacturer. Total RNA (0.5 µg) was reverse transcribed with 0.5 µg oligo-dT using M-MLV (Invitrogen). First-strand cDNA was used for real-time PCR with the Light cycler instrument, except for *Neurog3*, and FastStart DNA Master SYBR Green I kit (Roche). Primers were designed with Primer3 software (primer3_www.cgi v 0.2 – Whitehead Institute/MIT, Cambridge, MA, USA) and were as follows: HES1-FW: 5'-GAAAGATAGCTCCCGGCATT-3'; HES1-RV: 5'-ACCT-CGTTTCATGCACTCGC-3'; PDX1-FW: 5'-CATCTCC-CCATACGAAGTGC-3'; PDX1-RV: 5'-TGTAGGCAG-TACGGGTCTC-3'; ONECUT1-FW: 5'-AGACCTTC-CGGAGGATGTG-3'; ONECUT1-RV: 5'-TTGGACG-GACGCTTATTTTC-3'; FOXA2-FW: 5'-GACATACC-GACGCAGCTACA-3'; FOXA2-RV: 5'-GAGAAAGCA-GTCGTTGAAGG-3'; HPRT1-FW: 5'-TGTTGTTG-GATATGCCCTTG-3'; HPRT1-RV: 5'-AACTTGCGCT-CATCTTAGGC-3'. Serially diluted cDNA samples or PCR products were used to calculate the efficiency of each PCR with RealQuant software (Roche). After an initial Taq activation at 95 °C for 10 min, Light cycler PCR was performed using 45 cycles with the following cycling conditions: 95 °C for 15 s, 62 °C for 5 s, and 72 °C for 16 s. The *Hprt1* transcript was used as internal standard to control the amplification variations.

For real-time PCR of *Neurog3*, we used the primers as followed: NEUROG3-FW 5'-TTGCCCCACAACAT-CATCTG-3'; NEUROG3-RV 5'-TTGGGAGACTGGG-GAGTAGA-3', cyclophilin A-FW 5'-CAGGTCCTGGCA-TCTTGTTTC-3'; cyclophilin A-RV 5'-TTGCTGGTCT-TGCCATTCCCT-3' using a SYBR Green PCR master Mix, Applied Biosystem (Foster City, CA, USA) according to the instructions of the manufacturer. For both PCR analyses, the mean of the control samples was set as 1 to normalize the results.

Statistical analysis

All values are expressed as means ± S.D. Statistical significance was determined by using the two-tailed unpaired Student's *t*-test, and differences were considered to be statistically significant when *P* < 0.05.

Results

Menin is expressed in the developing pancreas

To investigate the role of menin in pancreas development in the mouse, we first analysed the menin expression pattern in

embryonic pancreata of wild-type mice at different developmental stages respectively at E11.5, E12.5, E13.5, E15.5, E16.5 and E18.5. Menin protein was detected by immunostaining with anti-menin antibodies in the entire pancreatic epithelium and surrounding tissues throughout the developmental stages (Fig. 1A–F). To ensure that menin is expressed in both endocrine and ductal cells, which constitute minor cellular compartments in pancreata, we performed IF staining of menin in combination with the pancreatic cell-specific markers on E16.5 pancreata. The results showed that menin was detected in insulin- and glucagon-expressing endocrine cells as well as Mucin1-expressing ductal cells (Fig. 1G–I), indicating that menin is expressed in these cell populations in developing pancreas.

Reduced number of glucagon-expressing cells in pancreata associated with apoptosis in E12.5 Men1 null embryos

We performed histological and IHC analyses on E12.5 pancreata, prior to the embryonic lethality of *Men1* null embryos (Bertolino *et al.* 2003a, Crabtree *et al.* 2003). At this stage, pancreases undergo the first transition of endocrine cell differentiation and display glucagon-positive cells. The

viability of all the analysed embryos was assessed by heart-beating. Both histological and IHC analyses were initially carried out at more than three different positions for each embryo. For those embryos where no glucagon staining was detected, the remaining tissues have been further subjected to the same analysis to confirm the result. The whole pancreata appeared histologically normal in E12.5 *Men1* null embryos (Fig. 2B and C), the branching of pancreatic bud rudiment being not significantly different from that seen in control embryos (Fig. 2A). However, the glucagon-expressing cells were either absent, in four out of seven E12.5 *Men1* null pancreata (Fig. 2E), or very few (Fig. 2F) in the remaining (three out of seven) compared with *Men1* wild-type embryos. These observations suggest that menin is required to support the differentiation or the survival of glucagon-expressing cells at this stage. To investigate whether the reduced number of glucagon-positive cells is associated with apoptosis, TUNEL assay was applied to analyse these developing pancreata. Indeed, apoptotic cells were detected in three out of four E12.5 *Men1* null embryos devoid of glucagon-positive cells, whereas they were completely absent in control embryos (Fig. 2G–L, Table 1). To further determine whether the absence of menin may trigger

Figure 1 Menin is expressed in the developing pancreas. Immunostaining of menin in developing pancreas at various stages: (A) E11.5, (B) E12.5, (C) E13.5, (D) E15.5, (E) E16.5, (F) E18.5. Insets show an amplified view of a part of pancreatic epithelium. The scale bars are 50 μm. (G–I) Double immunofluorescence of islet sub-populations of E16.5 pancreata using antibodies against menin (red), insulin (G, green), glucagon (H, green) and mucin-1 (I, green). The scale bars are 50 μm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0289>.

apoptosis in glucagon-positive cells, we carried out glucagon and TUNEL double staining. Indeed, a substantial proportion of glucagon-positive cells (70%) were found apoptotic in five *Men1* null pancreata having glucagon-positive cells (Fig. 2M–P). Such double-positive cells were not seen in four *Men1* wild-type control. These results suggest that menin prevents apoptosis in glucagon-secreting cells at this stage.

To gain insights into the molecular mechanisms responsible for the reduced number of glucagon-positive cells in *Men1* null embryos at E12.5, we examined the expression of several key genes in pancreas development, including *Hnf3 β* (Lee *et al.* 2001), currently called *Foxa2*, *Onecut1* (Jacquemini *et al.* 2000), *Pdx1* (Jonsson *et al.* 1994), *Hes1* (Jensen *et al.* 2000) and *Neurog3* (Gradwohl *et al.* 2000) by real-time PCR (Fig. 2Q and R) and immunostaining of PDX1 (Fig. 2S) and NEUROG3 (Fig. 2T). We found no detectable alteration of their expression in E12.5 *Men1* null pancreata analysed, suggesting that the role played by menin in the glucagon-positive cells at this stage is not likely via regulating gene expression of these factors.

Men1 null mutant ES cells failed to contribute normally to the formation of the pancreatic endocrine cells in chimeric mice

To determine whether the role of menin in the development of the pancreatic endocrine cells was due to the cell autonomous effects of menin, we next examined the capacity of *Men1* null ES cells to differentiate into the pancreatic endocrine cells by chimerism assay. To this end, two *Men1* wild-type and two *Men1* null ES cell lines were independently established from blastocysts derived from inter-crosses of heterozygous *Men1* mutant mice. After ascertaining their karyotype and ensuring that their gene expression profile corresponded to totipotent ES cells (Supplemental Fig. 1, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol199/issue2/>), they were injected into *Men1* wild-type C57BL/6 blastocysts to generate chimeric mice. The proportion of *Men1* null ES cell derivatives in the pancreatic islets of 3-week-old chimeric mice was then assessed by IHC (Fig. 3B–F and K) and DNA genotyping respectively (Fig. 3L). IHC analysis of six chimeric mice generated with *Men1* null ES cells using anti-menin antibodies revealed that, while mixed populations of menin-positive and -negative cells could be detected in the pancreatic exocrine compartment and in adjacent tissues (Fig. 3B and C), the islets were mainly composed of menin positively stained cells (Fig. 3D–F). Quantification of the chimerism by IHC analysis showed that more than 85% of islets contain more than 80% of menin-positive cells (Fig. 3K). In these islets, the lack of *Men1* null ES cell derivatives was seen both in α - and β -cells, as demonstrated by double IF staining (Fig. 3G–J). The data indicate that the majority of *Men1*

null ES cells failed to contribute to the formation of pancreatic islet cells.

In parallel, the contribution of *Men1* null ES derivatives in the islets of five chimeric mice was evaluated by quantifying the targeted *Men1* allele using real-time PCR. Compared with the skin, arbitrarily set as the reference tissue, the amount of *Men1* null ES derivatives in the islets is reduced 42%, and is only 47% of that found in the exocrine pancreas (Fig. 3L). Taken together, the chimerism assay revealed the impaired capacity of *Men1* null ES cells to differentiate *in vivo* into the pancreatic endocrine cells, albeit the presence of neighbouring *Men1* wild-type cells. These data indicate a cell-specific autonomous effect of menin in controlling pancreatic endocrine cell development. This observation, in consistence with our previous work (Bertolino *et al.* 2003a), also shows that lack of the *Men1* gene has no ubiquitous effect on the development of different cell types analysed, and supports the hypothesis that menin is specifically involved in endocrine development in the pancreas.

Menin is required for the normal development of the pancreatic endocrine, but not exocrine cells in pancreatic bud cultures

The embryonic lethality of *Men1* null embryos after E12.5 hampers the investigation of menin's role in the second transition of the endocrine pancreas. Therefore, to address this issue, we performed the pancreatic bud culture, which allows recapitulating of the second transition of endocrine cell differentiation (Duvillie *et al.* 2003, 2006, Attali *et al.* 2007). It was previously established that the cultured pancreases can develop exocrine and endocrine cells in the defined conditions, and the *in vitro* expression pattern of the major transcription factors found in the cultured pancreases, including NKX6-2, NEUROG3, PAX4, NEUROD1 and the markers of the mature β -cells PCSK1, PCSK2 and ABCC8, were similar to that found *in vivo* (Attali *et al.* 2007). Two independent series of pancreatic bud cultures using E12.5 *Men1* wild-type ($n=7$) and *Men1* null ($n=6$) heart-beating embryos were carried out. The development of endocrine and exocrine cells was evaluated after 7 days culture. Insulin/CPA1 double immunostaining revealed that, compared with the controls, *Men1* null pancreatic buds exhibited a significant reduction in the number of insulin-positive cells, while CPA1 staining for exocrine cells in the same sample was normal, indicating that the absence of menin resulted in failure of endocrine cell development without affecting that of exocrine pancreas (Fig. 4A–H and Q). Importantly, by insulin/glucagon double immunostaining, we found that the development of both α - and β -cells was clearly affected in *Men1* null cultured pancreatic buds (Fig. 4I–P, and R), indicating that the need of menin is therefore not restricted to a specific type of pancreatic endocrine cells. These findings are consistent with the observation made by IHC and IF analysis in chimeric mice.

Table 1 Abnormal development of the pancreatic endocrine cell in E12.5 *Men1* null mutant embryos. Summary of the results obtained from glucagon detection and TUNEL analysis.

	Glucagon staining	Apoptosis
<i>Men1</i> ^{+/+}		
1	+	—
2	+	—
3	+	—
4	+	—
5	+	—
6	—	—
<i>Men</i> ^{Δ/Δ}		
1	Weak	—
2	Weak	—
3	Weak	—
4	—	—
5	—	+
6	—	+
7	—	+

Six control (*Men1*^{+/+}) and seven *Men1*^{Δ/Δ} embryos were analysed and are shown.

Lack of menin results in altered structure and aberrant cell differentiation in cultured buds

We noticed that a substantial proportion (seven out of nine) of the *Men1* null pancreatic buds cultured at day 7 formed cystic structures that varied in size in different cultured buds (Fig. 5A–C). Similar cysts were not seen in *Men1* wild-type control (Fig. 5A). The formation of cystic structures in pancreas is considered to be a consequence of abnormal cellular differentiation of either ductal or endocrine cells (Cano *et al.* 2004, Pierreux *et al.* 2006). Indeed, we found that cells lining the cysts in *Men1* null pancreatic bud culture were negatively stained with the antibodies against PDX1 and Mucin-1 respectively the mature pancreatic β-cell and ductal cell markers (Fig. 5D–F and G–L). Further analysis showed the lack of both insulin- and glucagon-secreting cells within large cystic structures in cultured *Men1* null pancreatic buds at day 7 (Supplemental Fig. 4, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol199/issue2/>). All these data indicate that the cysts are delineated by an epithelium that follows neither mature α- and β-cell nor ductal cell fate. Our work demonstrates that the lack of menin altered the cellular differentiation of the pancreatic cells.

Markedly decreased number of *Neurog3*-expressing cells in the absence of menin in day 2 *Men1* null pancreatic bud cultures

To investigate the possible molecular and cellular events leading to the aberrant pancreatic endocrine cell development in the absence of menin in bud culture, we analysed the number of cells expressing *Neurog3*, a key factor for the pancreatic endocrine cell development (Gradwohl *et al.* 2000) after 2 days of culture, where its expression reaches the highest level in normal cultured buds. We found that the number of *Neurog3*-expressing cells was markedly decreased in day 2 *Men1* null pancreatic bud cultures ($n=5$) compared with *Men1* wild-type controls ($n=4$; Fig. 6A–E). On the other hand, neither the cell proliferation analysed by BrdU staining (Fig. 6F and G), nor the cell death determined by TUNEL assay (Fig. 6H–M) showed any difference between *Men1* null and wild-type cultured pancreatic buds at this time point. We also noticed that there was no obvious morphological alteration in day 2 *Men1* null cultured pancreatic buds (data not shown). The abnormal pancreatic endocrine cell development and the decreased number of *Neurog3*-expressing cells in cultured *Men1* null pancreatic buds are reminiscent of the phenotype of *Neurog3* mutant mice (Gradwohl *et al.* 2000). The data suggest that the reduced *Neurog3*-expressing cell number in the *Men1* null pancreatic bud cells could be the primary event leading to the observed defects.

Discussion

Based on its complete inactivation in MEN1 tumours, menin is considered to play a critical role in the control of cell growth in adult endocrine cells affected in the disease, including pancreatic endocrine α-, β- and PP cells, whereas its *in vivo* physiological function in these cells remains to be elucidated. Using different genetic approaches, we show that menin is a novel factor essential for the development of the pancreatic endocrine cells, including both α- and β-cells. We also demonstrate that menin is required neither for the formation of the pancreatic buds nor for development of the exocrine pancreas, indicating clearly that the effect of menin in the pancreas is endocrine cell specific. More importantly, chimerism analysis demonstrates that the presence of surrounding menin-expressing cells is not sufficient to allow

Figure 2 Reduced number of glucagon-positive cells in pancreatic bud associated with apoptosis in E12.5 *men1* null embryos. (B–F) Microscopic images of *Men1*^{Δ/Δ} and (A and D) *Men1*^{+/+} control pancreata sections stained with (A–C) HE and (D–F) antibodies against glucagon. Arrowheads indicate cells expressing glucagon. The scale bars are 50 μm. Microscopic images of *Men1*^{Δ/Δ} and *Men1*^{+/+} control pancreata sections stained with DAPI (G and H, blue), for apoptosis by TUNEL assay (I and J, green) and merged images (K–L). Arrowhead shows TUNEL-positive signal. Insets show an amplified view of a part of pancreatic bud. The scale bars are 50 μm. (M–P) Representative double TUNEL and glucagon staining of *Men1*^{Δ/Δ} ($n=5$) and control pancreata (*Men1*^{+/+}, $n=4$) serial sections from two independent experiments. The scale bars are 24 μm. (Q) Quantitative RT-PCR analysis of gene expression of *Pdx1*, *Foxa2*, *Onecut1* and *Hes1* in E12.5 *Men1* wild-type (*Men1*^{+/+}; white bar, $n=6$) and *Men1* null (*Men1*^{Δ/Δ}; black bar, $n=5$) pancreata. *Hprt1* was used as an internal control. (R) Quantitative RT-PCR analysis of *Neurog3* expression in E12.5 *Men1* wild-type (*Men1*^{+/+}; white bar, $n=4$) and *Men1* null (*Men1*^{Δ/Δ}; black bar, $n=4$) pancreata. An E15.5 *Men1* wild-type pancreata was used as the positive control (hatched bar). Cyclophilin was used as an internal control. (S) Immunohistochemical and (T) immunofluorescence analyses of E12.5 *Men1* wild-type (*Men1*^{+/+}) and *Men1* null (*Men1*^{Δ/Δ}) pancreas sections stained with the antibody against PDX1 (S) and NEUROG3 (T). The scale bars are 50 μm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0289>.

Figure 3 *Men1* null mutant ES cells fail to contribute to the formation of the pancreatic endocrine cells in chimeric mice. (A–F) Immunohistochemistry analysis of contribution of *Men1*^{+/+} cells to endocrine pancreas analysed by menin antibody on 3-week-old chimeric mice. The scale bars are 50 μ m. (A) Pancreas of control mice generated by micro-injection of *Men1* wild-type ES cells. (B–G) Chimeric mice generated with micro-injection of *Men1*^{+/+} ES cells: (B) exocrine pancreas, (C) intestine, (D–F) endocrine pancreas of three different chimeric mice. Note that the exocrine tissue shows a mixture of menin-negative and -positive cells. (G–J) Double immunofluorescence of pancreas of (G and I) control and (H and J) chimeric mice, showing the lack of *Men1* null ES cell derivatives in islets from the latter in both in α - and β -cells. The scale bars are 50 μ m. (G–H) Double staining with menin (red) and insulin (green). (I–J) Double staining with menin (red) and glucagon (green). (K) Quantification of menin-positive cells in the islets of six chimeric mice generated with micro-injection of *Men1*^{+/+} ES cells and stained by immunohistochemistry. To avoid a chimerism bias, only the islets situated in chimeric exocrine surroundings were scored. Between 6 and 15 (average 10) islets were scored for each chimeric mice. (L) The contribution of the *Men1*^{+/+} ES cell derivatives were analysed by the presence of the *Men1* targeted allele (*Men1*^{+/+}) in different tissues of five chimeric mice using real-time PCR, after normalization with the mouse *B2* gene as internal control. That of the skin is arbitrarily set as 1. Note that the contribution of the *Men1*^{+/+} ES cell derivatives to the liver is half of that to the skin (** $P < 0.01$), consistent with previous report (Bertolino *et al.* 2003a). Tissue abbreviations are as follows: Sk, skin; He, Heart; Exo. P., exocrine pancreas; Endo. P., endocrine pancreas; Li, Liver. Full colour version of this figure available via <http://dx.doi.org/10.1677/OE-08-0289>.

the *Men1* null ES derivatives to develop into the islet cells, indicating an intrinsic need of menin in the pancreatic endocrine cells. Finally, we found that the inactivation of the *Men1* gene affects the development of the pancreatic endocrine

cells before the second transition of cellular differentiation of these cells. Our data thus indicate the importance of menin in both the early glucagon-expressing endocrine cells and the initial steps of the major pancreatic endocrine cell

Figure 4 Menin is specifically required for normal development of the endocrine pancreas in pancreatic bud cultures. (A–H) Representative serial images of pancreatic bud cultures co-stained with antibodies against insulin (red) and carboxypeptidase A (CPA1, green). The scale bars are 100 μm. Note that the corresponding whole serial images are shown in Supplemental Fig. 2, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol199/issue2/>. (I–P) Representative serial images of pancreatic bud cultures co-stained with antibodies against insulin (red) and glucagon (green). The scale bars are 100 μm. Note that the corresponding whole serial images are shown in Supplemental Fig. 3, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol199/issue2/>. (Q–R) Quantification of positive staining signals in above immunostaining analyses in control (*Men1*^{+/+}, white bar, *n* = 7) and in *Men1* null pancreatic bud culture (*Men1*^{Δ/Δ}, black bar, *n* = 6) from two independent experiments. (Q) For each cultured bud, half sections were stained with insulin/CPA1 and insulin-positive signals were scored. (R) The other half was stained with insulin/glucagon and glucagon-positive signals were scored. The vertical axes represent mean value of the positive signals acquired for each bud (***P* < 0.01). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0289>.

Figure 5 *Men1* disruption results in altered pancreatic structure and aberrant cellular differentiation in day 7 cultured pancreatic bud. (A–C) Microscopic images of serial sections of day 7 (d7) cultured pancreatic buds respectively with H&E staining and (D–F) immunostaining with antibodies against PDX1. Insets show an amplified view of a part of stained pancreatic bud culture. The scale bars are 100 μm. Microscopic images of d7 cultured pancreatic buds by co-staining with DAPI (G and J, blue) and antibodies against Mucin-1 (green, H and K). Lower is the merge (I and L). The scale bars are 50 μm. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0289>.

Figure 6 Markedly reduced number of *Neurog3*-expressing cells in day 2 (d2) *Men1* null pancreatic bud cultures without alterations in cell proliferation or death. (A–D) *In situ* hybridization analysis of *Neurog3* expression in d2 cultured pancreatic buds. Two representative images from one *Men1*^{+/+} control (A and B) and one *Men1* null (*Men1*^{Δ/Δ}, C and D) bud are shown. The scale bars are 100 μ m. (E) *Neurog3*-positive signals in control (white bar, $n=4$) and in *Men1* null pancreatic bud culture (black bar, $n=5$) were scored in all sections of each. The vertical axis represents mean value of the positive signals obtained for each bud (** $P<0.01$). Note that the corresponding whole serial images for *Neurog3* *in situ* hybridization are shown in Supplemental Fig. 5, see Supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol199/issue2/>. (F–G) Microscopic images of d2 (F) *Men1*^{+/+} and (G) *Men1*^{Δ/Δ} cultured pancreatic buds with BrdU staining. Microscopic images of d2 (H–J) *Men1*^{+/+} and (K–M) *Men1* null (*Men1*^{Δ/Δ}) cultured bud sections stained with DAPI (blue, H and K), analysed by TUNEL assay (green, I and L) and merged images (J and M). Insets show an amplified view of a part of pancreatic bud. The scale bars are 50 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0289>.

development. However, the contribution of menin to the later stage of the major endocrine pancreas development could not be assessed in the current work. This would require either the application of siRNA in late wild-type pancreatic bud explants or temporally inducible disruption of a floxed *Men1* allele. Also, we noticed that a small number of islets from chimeric mice injected with *Men1* null ES cells, less than 2% of examined islets, show a near-equal proportion of menin-positive and -negative cells. This, along with the observed presence of diminished, but not entirely absent, glucagon-secreting cells in some of the E12.5 *Men1*^{-/-} embryonic pancreas, may suggest that the requirement of menin is either not absolute or can be biased by the occurrence of altered activities of other factors upon menin inactivation.

The reduced number of glucagon-expressing cells and the apoptotic glucagon positive cells were seen in E12.5 *Men1* null pancreata (under the first endocrine cell differentiation transition), suggesting that menin could play a role in cell survival in these cells at this early developmental stage.

However, we did not observe any alteration of cell death in *Men1* null pancreata cultured for 2 days, indicating that menin's role may imply different cellular functions in the second endocrine cell differentiation transition. Indeed, our data strongly suggest that menin is needed for the formation of *NEUROG3*-expressing cells during the crucial step of the second pancreatic transition where the progenitor cells commit to endocrine differentiation. We thus attribute the default of endocrine development in the cultured *Men1* null embryos to the lack of activated *NEUROG3* expression rather than cell death.

Transgenic lineage tracing studies demonstrated that the early glucagon-expressing cells are unlikely to be the progenitors of later mature endocrine cells (Herrera 2000). Therefore, the developmental failure of the pancreatic endocrine cells at the second transition in the absence of menin cannot be simply explained by the lack of early glucagon-expressing cells. Conversely, we noticed that cystic structures appeared in cultured *Men1* null pancreatic buds, which are delineated with

aberrantly differentiated cells. Considering the occurrence of deficient endocrine pancreas development, the formation of cystic structures in *Men1* null pancreatic bud cultures could be the consequence of abnormal cellular differentiation of cells lacking menin expression.

We did not detect any altered expression of major genes involved in the control of early pancreas development in E12.5 *Men1* null pancreata, including *Pdx1* (Jonsson *et al.* 1994), *Foxa2* (Lee *et al.* 2001), *Onecut1* (Jacquemin *et al.* 2000) and *Hes1* (Jensen *et al.* 2000), as well as *Neurog3*. However, the number of *Neurog3*-expressing cells, fated to become endocrine cells during the second endocrine cell differentiation transition (Edlund 2002, Wilson *et al.* 2003), was severely decreased in day 2 *Men1* null pancreatic bud cultures. This discrepancy may be due to the different molecular mechanisms involved respectively in the first and the second pancreatic endocrine cell differentiation transitions. As menin is closely related to transcriptional regulation, it is plausible that menin is needed, directly or indirectly, for normal NEUROG3 expression at this second stage of endocrine pancreas development, by acting upstream of NEUROG3. Further studies are needed to determine in detail the role played by menin in regulating NEUROG3 expression and the fate of NEUROG3-expressing cells. We noticed that none of the currently known protein partners of menin has been reported to be involved in the regulation of the *Neurog3* gene. Recently, Smart *et al.* (2006) has reported that the TGFβ1 pathway plays an important biological role in pancreas development, but mainly in maturation of differentiating β-cells. Obviously, the interaction of menin with SMAD proteins alone is not sufficient to explain its role found in this study, although we cannot exclude the possibility that it may participate in the TGFβ1 pathway at the later stage of pancreas development.

Among numerous factors implicated in the development of the pancreatic endocrine cells, *MEN1* is perhaps the first gene that is identified to be involved both in development and tumorigenesis of pancreatic endocrine cells. The seemingly contradictory biological effects of menin during embryonic development and the adult life of pancreatic endocrine cells indicate that menin plays fundamental but different roles during these two periods. This is reminiscent of another tumour suppressor, the *Rb* gene, which is not only responsible for the occurrence of retinoblastoma when inactivated, but also critically required for normal retinal development during the embryogenesis (MacPherson *et al.* 2004). Our data are also in consistency with the recent findings revealing multifaceted biological functions of menin in different cells and tissues reported by other groups (Sowa *et al.* 2003, Yokoyama *et al.* 2005). In particular, Sowa *et al.* (2003) have demonstrated that menin is required for the commitment of mesenchymal stem cells into the osteoblast lineage. By addressing the mechanisms of menin's essential function in the development of the pancreatic endocrine cells, one may know better physiological functions of menin, especially those implicated in the control of cellular differentiation and proliferation in endocrine cells. On the other hand, albeit identification of numerous factors

responsible for the development of the pancreatic endocrine cells, many biological aspects of these cells remain to be elucidated. The identification of menin's role in this procedure should be of help not only for a better understanding of these cells, but also for the development of new therapeutic strategies for the treatment of the diseases affecting the pancreatic endocrine cells, including both tumours and metabolic disorders.

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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