0888-8809/03/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 17(9):1880–1892

doi: 10.1210/me.2003-0154

Heterozygous *Men1* Mutant Mice Develop a Range of Endocrine Tumors Mimicking Multiple Endocrine Neoplasia Type 1

PHILIPPE BERTOLINO, WEI-MIN TONG, DOMINIQUE GALENDO, ZHAO-QI WANG, AND CHANG-XIAN ZHANG

Laboratory of Genetics (P.B., C.-X.Z.), Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5641, Faculty of Medicine University of Lyon 1, 69373 Lyon, France; and International Agency for Research on Cancer (P.B., W.-M.T., D.G., Z.-Q.W.) 69008 Lyon, France

Multiple endocrine neoplasia type 1 (MEN1) is a hereditary syndrome characterized by the occurrence of multiple endocrine tumors of the parathyroid, pancreas, and anterior pituitary in patients. To study tumorigenesis related to the MEN1 syndrome, we have generated *Men1* knockout mice using the gene targeting approach. Heterozygous *Men1* mutant mice developed the same range of major endocrine tumors as is seen in MEN1 patients, affecting the parathyroid, pancreatic islets, pituitary and adrenal glands, as well as the thyroid, and exhibiting multistage tumor progression with metastatic potential. In particular, extrapancreatic gastrinoma, pancreatic glucagonoma, and mixed hormone-producing tumors in islets were observed. In addition, there was a high incidence of gonadal tumors of endocrine origin, *i.e.* Leydig cell tumors, and ovary sex-cord stromal cell tumors in heterozygous *Men1* mutant mice. Hormonal disturbance, such as abnormal PTH and insulin levels, was also observed in these mice. These tumors were associated with loss of heterozygosity of the wild-type *Men1* allele, suggesting that menin is involved in suppressing the development of these endocrine tumors. All of these features are reminiscent of MEN1 symptoms in humans and establish heterozygous *Men1* mutant mice as a suitable model for this disease. (*Molecular Endocrinology* 17: 1880–1892, 2003)

THE *MEN1* GENE, which codes for the protein menin, has been identified as being responsible for human multiple endocrine neoplasia type 1 (MEN1, OMIM 131100), a hereditary syndrome transmitted as an autosomal dominant trait (1, 2). The disease is characterized by the occurrence of multiple endocrine tumors of the parathyroid, endocrine pancreas, and anterior pituitary, as well as foregut endocrine carcinoids and adrenal cortical tumors. Several nonendocrine tumors have also been seen in patients suffering from MEN1, such as lipoma and angiomas. Although complex in its clinical manifestation, the MEN1 syndrome can be distinguished from other polyendocrinopathies as a clinical entity by its special tumor spectrum (3, 4).

Germline mutations in the *MEN1* gene have been detected in about 85% of familial MEN1 cases (5–7) and somatic mutations have also been found in several types of sporadic endocrine tumor, especially sporadic parathyroid adenomas (8), gastrinomas and insulinomas (9). Both germline and sporadic mutations show a typical loss of function profile, with different types of mutation detected along the whole coding sequence (4, 10). However, these observations do not establish any genotype-phenotype correlation, and say very little about the potential importance of the gene in tumor development. In addition, loss of heterozygosity (LOH) has been observed both in familial MEN1 tumor tissues and in their sporadic counterparts, suggesting the tumor suppressing nature of the *MEN1* gene (11–13).

To examine the role of menin in tumor development, the murine homolog of the gene (*Men1*) was disrupted (14). In this mouse model, although homozygous mutant mice die at embryonic day E11.5-E12.5, heterozygous *Men1* mutant mice develop multiple endocrine tumors, mainly in the pancreas, parathyroid, and less frequently in the adrenal gland (14). However, gastrinomas, the predominant MEN1 extrapancreatic tumor type, and pancreatic glucagonomas, were not reported. In addition, whether this *Men1* mouse model also shows hormonal disturbance of PTH, which is commonly seen in MEN1 patients, is not known. In the present study, we report a novel mouse model showing that heterozygous *Men1* mutant mice develop a full range of multiple endocrine tumors and symptoms, including extra-pancreatic gastrinoma, pancreatic glucagonoma, adrenal and thyroid tumors, as well as disturbed hormone secretion. We also found a high incidence of gonadal tumors of endocrine origin, Leydig cell tumors, and sex-cord stromal cell tumors in these heterozygous *Men1* mutant mice. These tumors exhibit LOH of the wild-type *Men1* allele. Finally, these

Abbreviations: EF, Embryonic fibroblast; HSD, hydroxysteroid dehydrogenase; LOH, loss of heterozygosity; MEN1, multiple endocrine neoplasia type 1; PRL, prolactin; RPA, replication protein A.

mice also develop thyroid tumors that are associated with LOH of *Men1*, providing experimental evidence to a potential link between thyroid tumors in MEN1 patients and loss of function of menin.

RESULTS

Men1 Heterozygous Mutant Mice Develop Multiple Endocrine Tumors

Using the gene targeting technique, we disrupted the *Men1* gene in mice (15). Homozygous mutant (*Men1*$^{T/T}$) mice died at E11.5-E13.5 with multiple developmental defects, including nonclosure of the neural tube, heart hypotrophy and altered organization of the epithelial and hematopoietic compartments in the liver. However, heterozygous mutant (*Men1*$^{+/T}$) mice appeared normal and did not show any phenotypic abnormalities at a young age (15).

Because MEN1 patients heterozygous for the loss-of-function mutation in *MEN1* develop multiple endocrine neoplasia with a high penetrance in the parathyroid, pancreas, pituitary, and adrenal glands (3), we examined the *Men1*$^{+/T}$ mice for tumors in these organs. We performed histological and molecular analyses on the major organs of these mice and found that the mice developed hyperplastic lesions in the pancreas as early as eight months of age. Around 12–13 months of age, a variety of dysplastic and tumor lesions in the parathyroid, pancreas, pituitary and adrenal gland were observed. In *Men1*$^{+/T}$ mice older than 13 months, a high incidence of multiple endocrine tumors was noted, and nearly all mice developed more than one endocrine tumor (Table 1, not shown).

Parathyroid Tumors

Although wild-type mice developed no morphological abnormalities in the parathyroid, adenomas were first found in this organ in *Men1*$^{+/T}$ mice at 12 months of age. With aging, parathyroid adenomas were detected in seven of 17 (41%) *Men1*$^{+/T}$ mice in the 13- to 18-month age group and 21 of 33 (64%) mice over 19 months (Table 1, Fig. 1, A–C). We found no significant

Table 1. Tumor Development in *Men1*$^{+/+}$ and *Men1*$^{+/T}$ Mice

Organ/Tissue Type of pathology	8–12 Months		13–18 Months		19–26 Months	
	Wild type (n = 9)	*Men1*$^{+/T}$ (n = 23)	Wild type (n = 12)	*Men1*$^{+/T}$ (n = 36)	Wild type (n = 22)	*Men1*$^{+/T}$ (n = 61)
Parathyroid						
Dysplasia	0/5	0/5	0/12	1/17 (5.9%)	2/20 (10%)	0/33
Adenoma	0/5	1/5 (20%)	0/12	7/17 (41.2%)	0/20	21/33 (63.6%)
Carcinoma	0/5	0/5	0/12	0/17	0/20	1/33 (3%)
Extrapancreatic gastrinoma						
Adenoma	0/8	1/12 (8.3%)	0/5	2/20 (10%)	0/6	3/17 (17.6%)
Carcinoma	0/8	0/12	0/5	1/20 (5%)	0/6	1/17 (5.9%)
Pancreatic islets						
Hyper/dysplasia	0/9	15/23 (65.2%)	2/13 (15.4%)	13/34 (38.2%)	0/21	7/61 (11.5%)
Adenoma	0/9	5/23 (21.7%)	0/13	4/34 (11.7%)	0/21	12/61 (19.7%)
Carcinoma	0/9	2/23 (8.7%)	0/13	13/34 (38.2%)	0/21	37/61 (60.6%)
Pituitary						
Adenoma	0/7	1/23 (4.3%) F = 1/11; M = 0/12	0/8	2/31 (6.4%) F = 1/15; M = 1/16	0/20	10/60 (16.6%) F = 8/35; M = 2/25
Carcinoma	0/7	1/23 (4.3%) F = 1/11; M = 0/12	0/8	4/31 (12.9%) F = 3/15; M = 1/16	0/20	12/60 (20%) F = 8/35; M = 4/25
Adrenal glands						
Nodular hyperplasia	0/9	2/23 (8.7%)	0/11	7/31 (22.6%)	0/21	5/61 (8.2%)
Adenoma	0/9	3/23 (13%)	0/11	4/31 (12.9%)	0/21	17/61 (27.8%)
Carcinoma	0/9	0/23		0/31	0/21	11/61 (18%)
Testis Leydig cells						
Hyper/dysplasia	0/5	9/12 (75%)	1/7 (14.3%)	5/17 (29.4%)	0/9	2/25 (8%)
Tumor	0/5	3/12 (25%)	0/7	10/17 (58.8%)	1/9 (11.1%)	22/25 (88%)
Sex-cord stromal cells						
Tumor	0/4	0/9	0/5	5/16 (31.2%)	0/13	14/28 (50%)
Thyroid glands						
Hyper/dysplasia	0/9	0/23	0/12	0/36	0/22	4/61 (6.5%)
Tumor	0/9	0/23	0/12	0/36	0/22	4/61 (6.5%)
Mammary glands						
Carcinoma	N.D.	N.D.	N.D.	N.D.	0/13	3/36 (8.3%)

Data are shown as number of lesions/number of mice examined, with the percentage indicated in *parentheses*. n, Total number of mice analyzed. ND, Not determined; F, female; M, male.

difference in PTH secretion between a group of randomly selected *Men1*$^{+/T}$ mice aged 9–26 months (mean ± SE = 187.5 ± 31.66 pg/μl, n = 48) and the wild-type control group (144.8 ± 15.60 pg/μl, n = 22) (Fig. 1D). However, a few mice exhibited increased PTH levels, one having an increase of about 5-fold and two others a more than 10-fold increase, compared with the mean level of the control group (Fig. 1D).

Extrapancreatic G-Cell Hyperplasia and Gastrinomas

Among 49 *Men1*$^{+/T}$ mice examined, we observed gastrointestinal lesions in eight mice, mainly situated in the duodenum or at the junction of the glandular stomach and duodenum. No similar abnormalities were detected in the 19 wild-type mice included in the study (Table 1). Histological examination of these lesions revealed adenomas and carcinomas in the glandular stomach and duodenum (Table 1; Fig. 2, B and C). Specifically, tumors located in the mucosa of the bodyfunds glandular stomach were composed of uniform cells with scanty cytoplasma and invaded the submucosa layer (Fig. 2B). To further define whether these tumors were associated with gastrointestinal endocrine cells, we performed immunohistochemical analysis using an antibody against gastrin and found gastrinomas in the glandular stomach (Fig. 2E) or duodenum (Fig. 2F). These characteristics are reminiscent of gastrinomas in humans.

Pancreatic Islet Tumors

Hyperplastic islets were first detected at eight months of age. At 8–12 months of age, 15 of 23 (65%) mice contained hyperplastic or dysplastic islets, five (22%) developed islet adenomas and two (9%) carcinomas (Table 1). A marked increase in islet carcinomas (13 of 34 mice) was found at 13–18 months of age. It was also noted that enlarged tumor masses were often seen in the pancreas after 18 months of age (not shown). Of 61 *Men1*$^{+/T}$ mice analyzed, 37 (60%) developed multifocal advanced islet carcinomas. With the exception of three islet hyperplasias or dysplasias, no islet tumor was detected in the pancreas of any of the 43 *Men1*$^{+/+}$ mice during the period of 8–26 months (Table 1).

To define the cell of origin of *Men1*$^{+/T}$ islet tumors, 12 pancreases containing islet tumors were subjected to serial sectioning and analyzed for insulin, glucagon, and gastrin expression by immunohistochemical staining. The majority of the islet adenomas and car-

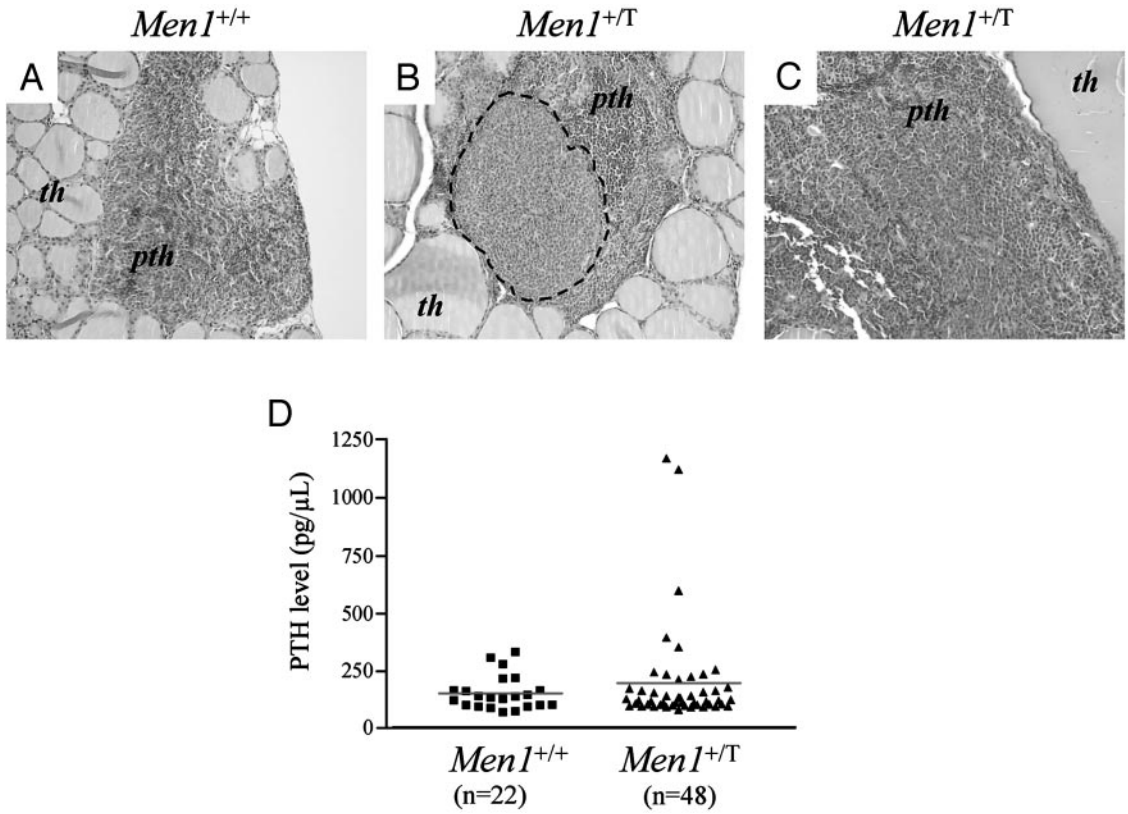


Fig. 1. Parathyroid Tumors Associated with Altered PTH Secretion in *Men1*$^{+/T}$ Mice

A, Normal parathyroid (*pth*) and thyroid (*th*) in wild-type mice; B, a parathyroid adenoma (*black hashed circle*) and (C) a carcinoma in *Men1*$^{+/T}$ mice are shown. Original magnification: ×20. D, Quantification of serum PTH levels with *bars* representing the mean value of all the individual measurements.

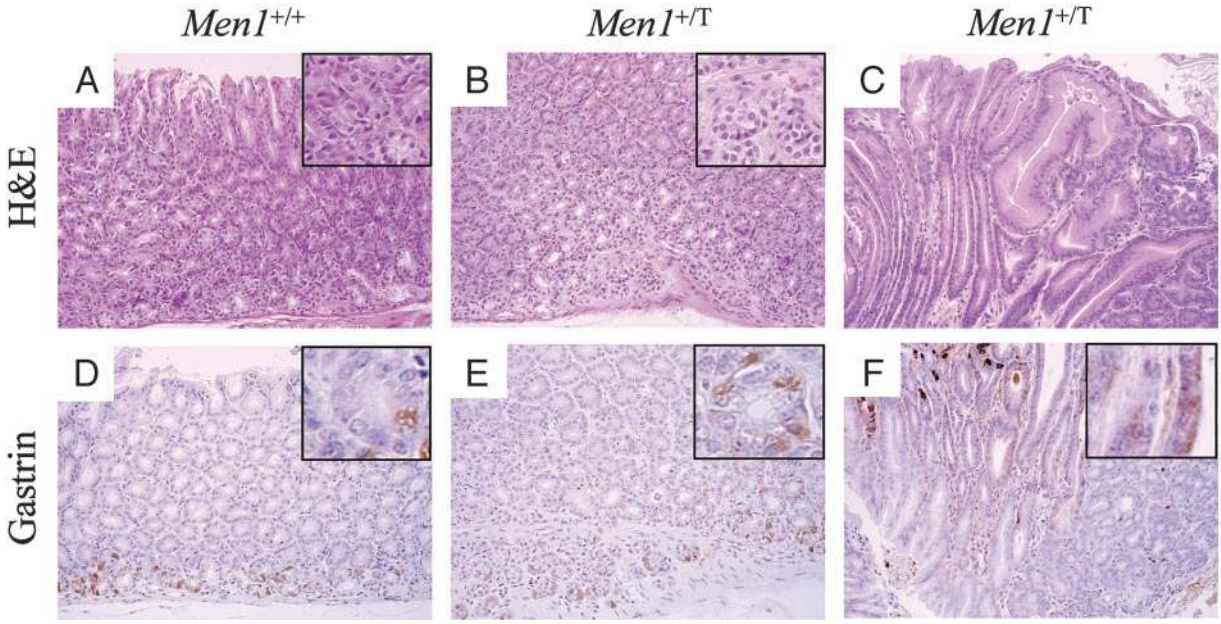


Fig. 2. Extrapancreatic Gastrinomas in *Men1*$^{+/T}$ Mice

Normal wild-type glandular stomach (A) with gastrin immunostaining (D), and gastrinomas derived from the glandular stomach (B and E) and duodenum (C and F) in *Men1*$^{+/T}$ mice are shown. Immunostaining shows gastrin positive cells in tumors (E and F). High magnification of gastrin positive cells are shown in *insets*. Original magnification, ×20.

cinomas found in these *Men1*$^{+/T}$ mice expressed high levels of insulin and were thus identified as β-cell insulinomas (Fig. 3F). However, a subset of islet tumors, seen in 6 of 12 mice, showed strong glucagon immunoreactivity (α-cell tumors or glucagonomas, Fig. 3K). During tumor progression, advanced islet carcinomas showed either weak insulin and glucagon expression, or a complete loss of their expression (not shown), suggesting a dedifferentiation process. However, these tumors usually lacked expression of gastrin (Fig. 3, H and L) and somatostatin (not shown). Interestingly, some islet tumors (seen in 4 of 12 mice analyzed) expressed two hormones simultaneously, such as insulin and glucagon (Fig. 3, J and K), or glucagon and gastrin (Fig. 3, O and P), suggesting a mixed hormone production. The existence of islet tumors that costained for both glucagon and gastrin in these *Men1*$^{+/T}$ mice is a characteristic of pancreatic gastrinomas displaying mixed hormone secretion in MEN1 patients (16).

To examine whether these islet tumors affected insulin levels in the bloodstream of *Men1*$^{+/T}$ mice, we quantified the serum insulin levels by an ELISA assay. The level of insulin was higher in *Men1*$^{+/T}$ mice (mean $\pm$ standard error = 2.49 $\pm$ 0.58 μg/liter, n = 17) than in age-matched wild-type controls (1.25 $\pm$ 0.23 μg/liter, n = 13), with high heterogeneity (0.72–10.56 μg/liter). Altogether, the *Men1*$^{+/T}$ mice developed multi-focal pancreatic islet tumors derived from insulin-, glucagon-, and gastrin-secreting cells, mimicking the pancreatic pathology in human MEN1 (3).

Pituitary Tumors

An enlarged pituitary was often found in *Men1*$^{+/T}$ mice beyond 13 months of age, compared with age-matched wild-type controls (Fig. 4, A and B). Histological examination revealed that almost all the pituitary tumors were localized in the *pars distalis* (Fig. 4, D and E), corresponding to the human anterior pituitary, the most commonly affected site in MEN1 patients. Six pituitary tumors were observed in 31 (19%) *Men1*$^{+/T}$ mice at 13–18 months of age, and 22 (37%) of 60 *Men1*$^{+/T}$ mice developed pituitary tumors after the age of 18 months (Table 1). These tumors were more common in females (79%), as reported for human pituitary tumors, including those of MEN1 patients (17). As pituitary tumors found in MEN1 patients are mainly characterized as prolactinomas and GH-secreting tumors (3, 17), we performed immunohistochemical staining using antibodies against GH and prolactin (PRL). Of a total of 15 *Men1*$^{+/T}$ pituitary tumors analyzed, six showed strong GH immunoreactivity (Fig. 4G), and nine were exclusively positive for PRL (Fig. 4K). However, all tumors were negative for ACTH staining (not shown).

Adrenal Cortical Tumors

Macroscopic examination revealed an enlargement of adrenal glands, often bilateral, in *Men1*$^{+/T}$ mice compared with age-matched wild-type controls (Fig. 5, A and B). Histological analysis of the 23 *Men1*$^{+/T}$ glands showed nodular hyperplasia in two (9%) and adeno-

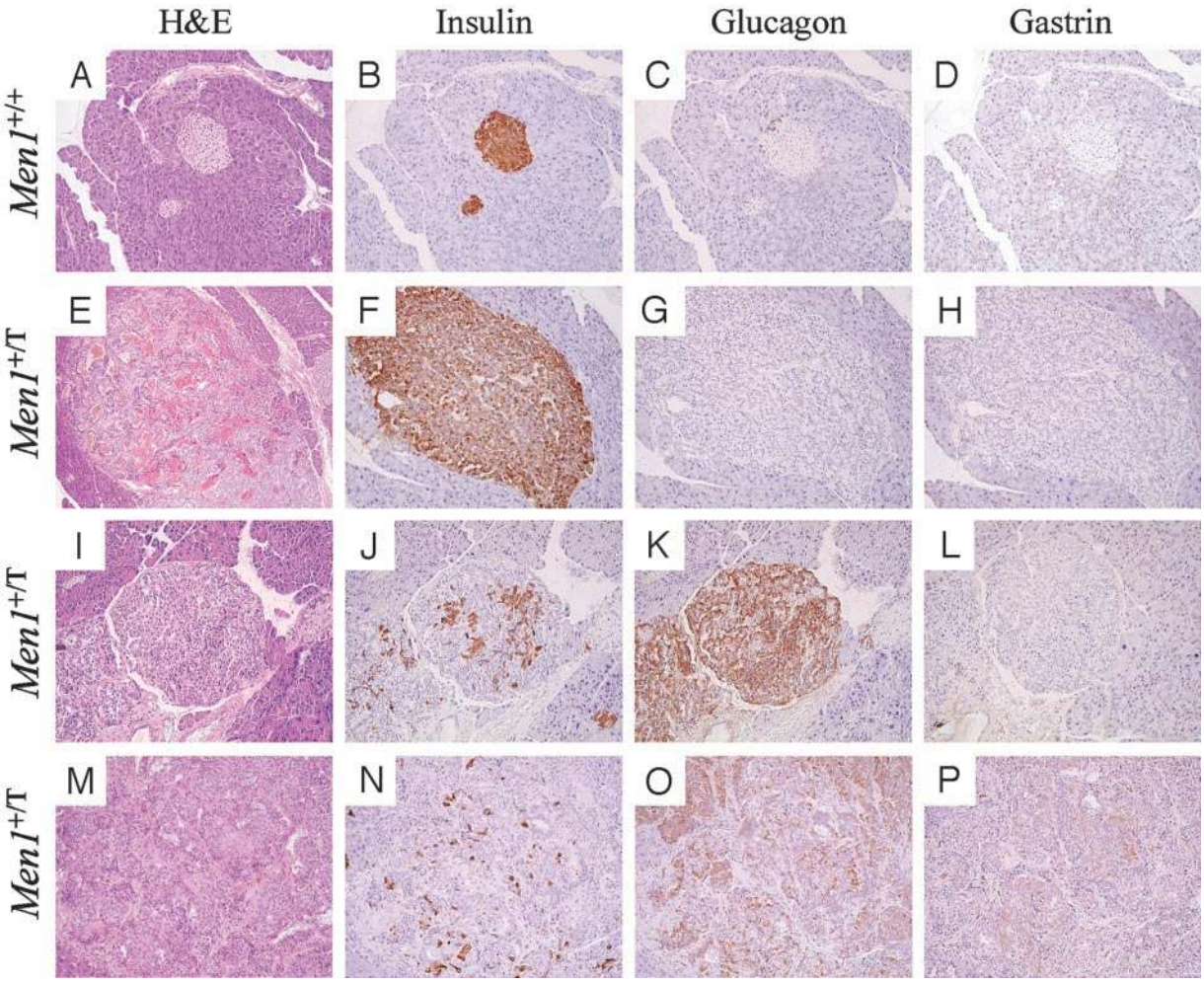


Fig. 3. Pancreatic Islet Tumors in *Men1*$^{+/T}$ Mice

Sections of the normal islet (A–D), insulinoma (E–H), and glucagonoma (I–L). Hematoxylin and eosin and immunostaining for insulin, glucagon, and gastrin were performed on serial sections. Note a mixed islet tumor expressing both insulin and glucagon (J and K) and a tumor positive for both glucagon and gastrin (O and P). Original magnification, ×20.

mas in three (13%) at 8–12 months of age (Table 1). At 13–18 months of age, 7 of 31 (23%) *Men1*$^{+/T}$ mice exhibited nodular hyperplasia and four (13%) developed adenomas. After 18 months, 28 of 61 (46%) *Men1*$^{+/T}$ mice developed adenomas or carcinomas in the adrenal cortex (Fig. 5D and Table 1).

Leydig Cell Tumors and Ovary Sex-Cord Stromal Cell Tumors

In addition to the endocrine tumors mentioned above, the heterozygous *Men1* mutant mice developed a high frequency of tumors in the gonad (Table 1). Gross examination revealed a marked increase in testicular and ovarian size in *Men1*$^{+/T}$ mice after 13 months of age compared with age-matched wild-type controls (not shown). In 12 male *Men1*$^{+/T}$ mice at 8–12 months of age, nine (75%) exhibited gonadal stromal hyperplasia or dysplasia and three (25%) stromal cell tumors. The tumor incidence reached 59% and 88% in the 13- to 18-month and 19- to 26-month age groups, respectively (Table 1). Histologically, the focal stromal hyperplasia (Fig. 6, B and E) and multinodular tumors (Fig. 6, C and F) were frequently bilateral, and compressed the surrounding seminiferous tubules, resulting in degenerative changes (not shown). Tumor cells were large and often polygonal, with an abundant granular eosinophilic cytoplasm and a central round nucleus. In addition, they occasionally contained lipid pigment and vacuoles (Fig. 6C, *inset*). To define the possible cell of origin of these tumors, we performed immunostaining using an antibody against 3β-hydroxysteroid dehydrogenase (3β-HSD), an enzyme specifically expressed in testis Leydig cells that is responsible for synthesis of androstenedione. In contrast to age-matched wild-type testes, where sporadic Leydig cells were found among the stromal cells (Fig. 6, A and D, *inset*), *Men1*$^{+/T}$ testes contained hyperplastic stromal cells and tumors, both with strong 3β-

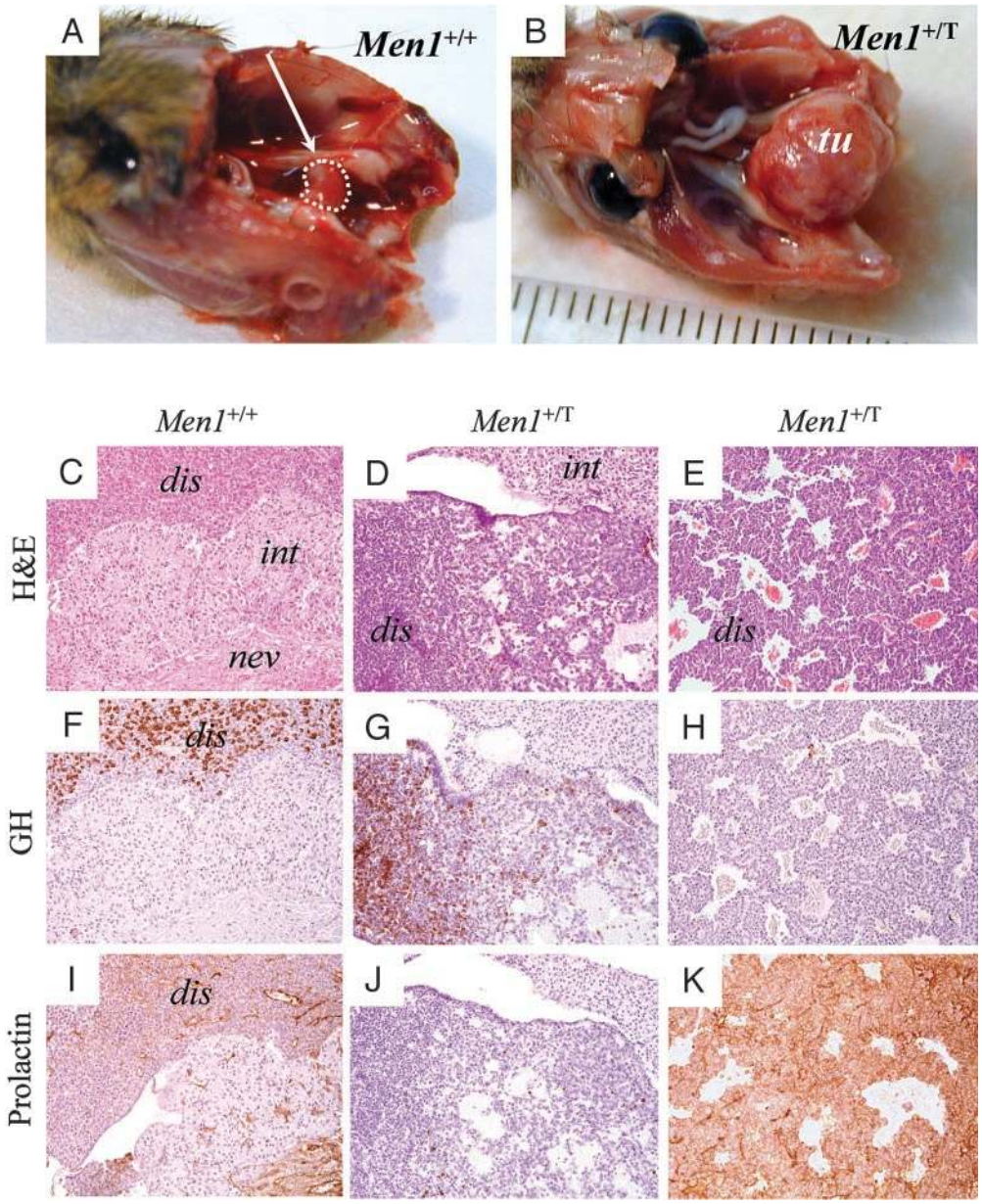


Fig. 4. Pituitary Lesions in *Men1*$^{+/T}$ Mice

Macroscopic view of the normal pituitary (*white arrow* and *hashed circle*) from a wild-type mouse (A) and a pituitary tumor mass (*tu*) from a *Men1*$^{+/T}$ mouse (B). Morphology of a normal pituitary (C) showing GH and PRL staining in the *pars distalis* (*dis*) (F, I). A representative *Men1*$^{+/T}$ pituitary tumor (D) exhibits GH staining (G) but is negative for PRL (J), whereas another one shows staining for PRL (K) but lacks GH expression (H). *dis, Pars distalis*; *int, pars intermedia*; *nev, pars nervosa*. Original magnification, ×20.

HSD expression (Fig. 6, E and F). These immunohistochemical data demonstrate that the Leydig cells represent the cell of origin of the testicular tumors.

We also found that 19 of 44 (43%) of *Men1*$^{+/T}$ females developed ovarian tumors between the ages of 13 and 26 months (Table 1, Fig. 6, H and I). Histological analysis of these tumors revealed that all were derived from sex-cord stromal cells, mainly granulosa cells, containing large round nuclei (Fig. 6I, *inset*). While a normal ovarian histological structure was partly preserved in these tumors, the follicular structure was largely replaced by the tumor mass (Fig. 6, H and I). Ovarian tumor cells also expressed high levels of 3β-HSD (not shown), indicating steroidogenesis in the tumor cells.

Thyroid Abnormalities and Other Rare Tumors in *Men1*$^{+/T}$ Mice

Besides the above tumors, other tumor types were identified at low frequencies in aged Men1$^{+/T}$ mice

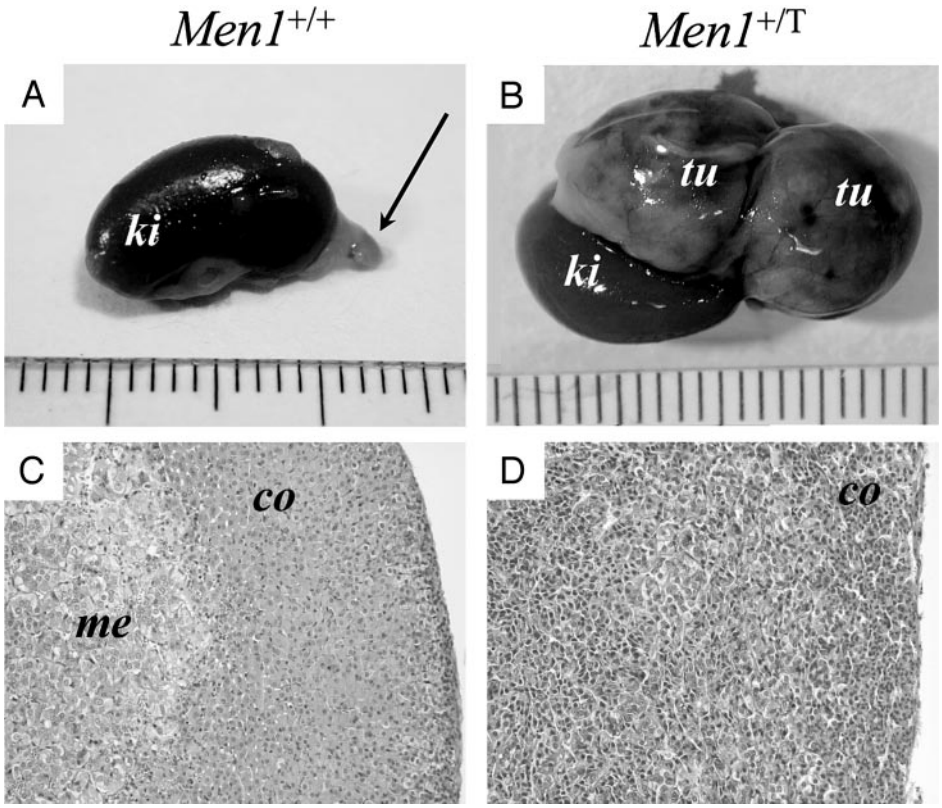


Fig. 5. Adrenal Tumors in *Men1*$^{+/T}$ Mice

Macroscopic views of a normal adrenal gland in wild-type mice (*arrow* in A) and tumors (*tu*) in *Men1*$^{+/T}$ mice (B). *ki*, Kidney. Normal structure of the adrenal gland (C) and a cortical tumor in a *Men1*$^{+/T}$ mouse (D). *me*, Medulla; *co*, cortex. Original magnification, ×20.

during our systemic histological screening (Table 1). Among 61 *Men1*$^{+/T}$ mice analyzed, thyroid abnormalities, including hyperplasia (6.5%; n = 4) and tumors (6.5%; n = 4), were found. Histological analysis revealed that thyroid follicular cell hyperplasia localized focally (Fig. 7, B and E), and solid follicular cell carcinomas contained tumor cells in densely packed nest and forming a few minute follicles (Fig. 7, C and F). Mammary gland carcinomas were also detected in 3 of 36 *Men1*$^{+/T}$ female mice over 18 months of age (Table 1).

Metastasis of Endocrine Tumors in *Men1* Heterozygous Mutant Mice

To investigate whether the endocrine tumors in *Men1*$^{+/T}$ mice metastasized, we histologically examined lymph nodes surrounding the endocrine organs and performed immunohistochemical analysis. Local lymph node metastasis of insulinomas from three of 50 *Men1*$^{+/T}$ tumors was evident (Fig. 8, A and B). Moreover, 10 of 16 (59%) pituitary tumors showed invasion into the brain stem, pressing against adjacent brain tissues (Fig. 8, C and D) and these tumor cells showed PRL staining (not shown). Finally, metastasis of Leydig cell tumors to local lymph nodes and distal organs such as the lung (Fig. 8, E and F) was observed in 7 of 42 (17%) *Men1*$^{+/T}$ males aged 13–22 months. These findings demonstrate the metastatic potential of *Men1*$^{+/T}$ endocrine tumors and confirmed that these tumors display a full spectrum of multistage tumor progression: hyperplasia, dysplasia, adenoma, and carcinoma, as well as metastasis.

LOH of the Wild-Type *Men1* Allele in Tumors

To investigate whether the development of multiple endocrine tumors in heterozygous *Men1* mutant mice was due to loss of the wild-type *Men1* allele, we carried out PCR and Southern blot analyses using DNA isolated from fresh tumors or from microdissected tumor samples of *Men1*$^{+/T}$ mice. We analyzed 34 tumors originating from the islet, pituitary, adrenal gland and gonad, as well as thyroid, and found either complete or partial loss of the wild-type *Men1* allele in all of them (Fig. 9, A and B). The remaining wild-type bands (in Fig. 9, A and B) in some samples may reflect contamination by normal cells in these tumor masses. Therefore, the development of multiple endocrine neoplasia in *Men1*$^{+/T}$ mice is most likely attributable to LOH of the *Men1* gene, consistent with the findings in human MEN1 tumors (11, 18).

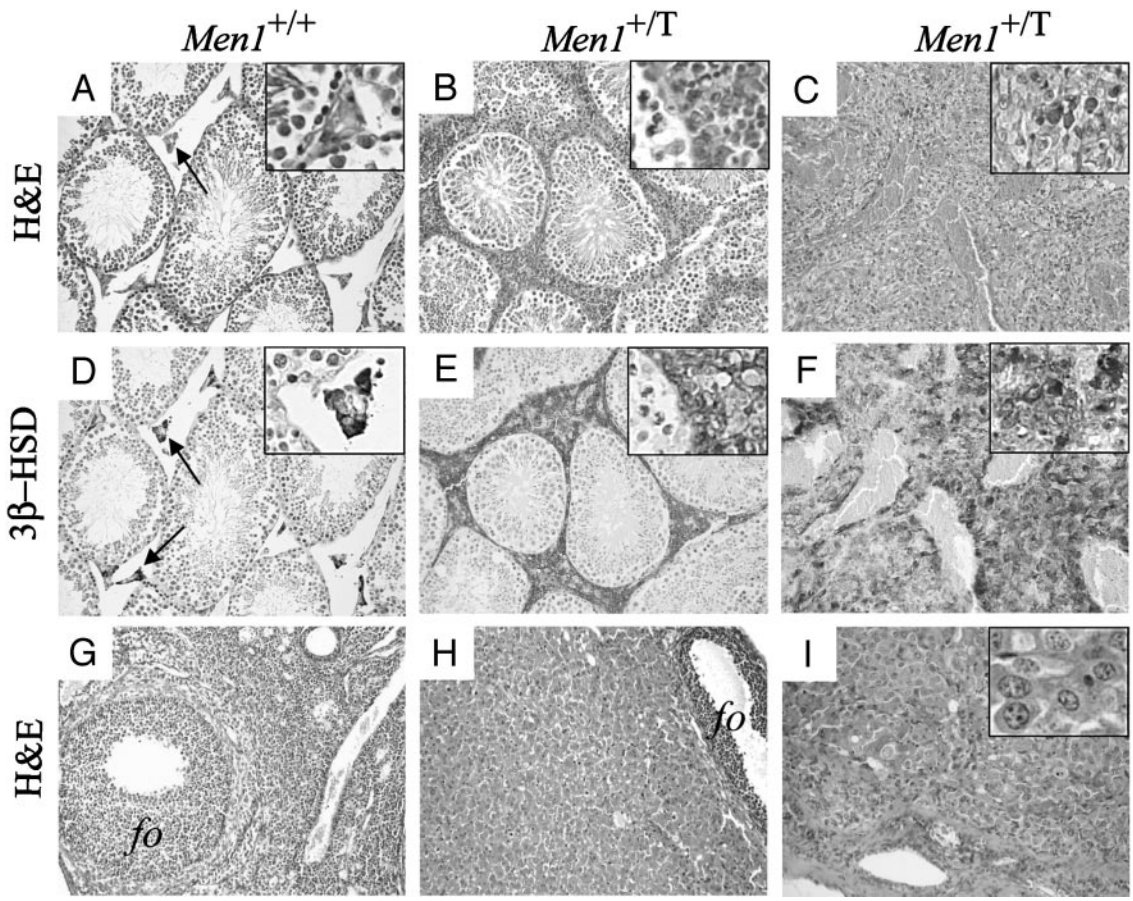


Fig. 6. Leydig Cell Tumors and Sex-Cord Stromal Cell Tumors in *Men1*$^{+/T}$ Mice

Normal Leydig cells are situated in the stroma in the wild-type testis (*arrow* in A) and express 3β-HSD (*arrows* in D). Leydig cell hyperplasia (B) and tumors (C) in *Men1*$^{+/T}$ mice show strong anti-3β-HSD expression (E and F). Normal morphology of a wild-type ovary (G), and sex-cord stromal cell tumors in *Men1*$^{+/T}$ mice (H and I). *Inset* in (I) shows large round nuclei of tumor cells. *fo*, Follicles. Original magnification, ×20.

DISCUSSION

In the present study, we have generated *Men1* knockout mice to investigate the function of menin in tumorigenesis. We found that heterozygous *Men1* mutant mice developed a high incidence of multiple endocrine tumors, affecting the parathyroid, pancreas, pituitary and adrenal glands as well as thyroids. In particular, gastrinomas, the most frequent enteropancreatic tumor encountered in about 50% of MEN1 patients (3, 4), and glucagonomas were both observed in these mice. The tumor spectrum of *Men1*$^{+/T}$ mice is remarkably similar to that seen in MEN1 patients. It appears that the hyperplasia and tumor lesions in the pancreas not only are multifocal, but also originated from different islet cells, reminiscent of the variable clinical symptoms and disturbances of hormone secretion in MEN1 patients. Other features of the MEN1 syndrome, such as the relative proportions of prolactinomas and GH-secreting adenomas in the pituitary, and the bilateral occurrence of cortical adenomas in the adrenals, were also developed in our model. We also observed a high prevalence of pituitary tumors in female mice, a well-known feature of human pituitary tumors including those found in MEN1 patients, suggesting that the factors underlying these tumors are probably similar in mice and humans.

In addition, the observation that *Men1*$^{+/T}$ mice developed thyroid abnormalities and the loss of the wild-type *Men1* allele was found in all four *Men1*$^{+/T}$ thyroid tumors analyzed is interesting. Although LOH at chromosome 11q and 11q13 in thyroid follicular neoplasms has been previously reported (19, 20), the association of thyroid abnormalities with MEN1 pathology was not established (Ref. 3; see also Ref. 14). Our results imply that complete inactivation of the *MEN1* gene is likely a crucial genetic event in thyroid tumorigenesis in MEN1 patients.

The tumor spectrum observed in the present study is wider than that previously reported (14). Some tumor types, such as gastrinomas, glucagonomas and gonadal tumors, were not reported in the previous study. We noticed a hormonal disturbance associated with the occurrence of parathyroid adenoma, the most

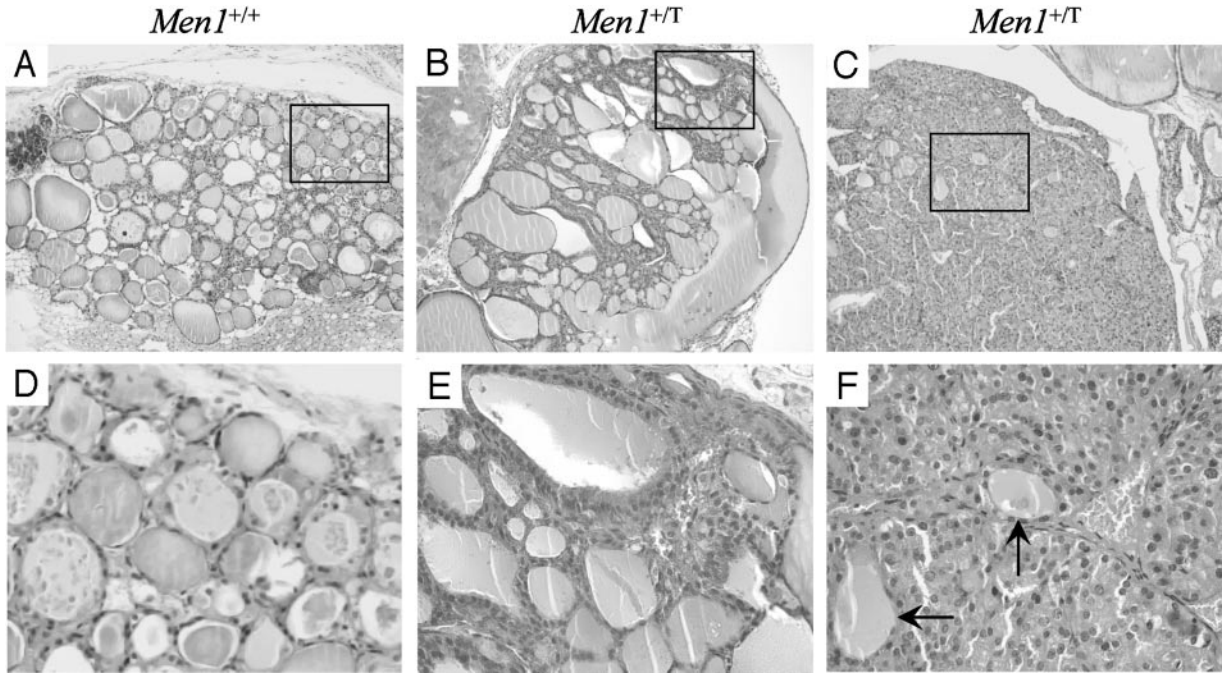


Fig. 7. Thyroid Tumors in *Men1*$^{+/T}$ Mice

A and D, Normal histology of the thyroid gland from a wild-type mouse. B and E, Thyroid follicular cell hyperplasia showing that the focal gland was replaced by hyperplastic tissues. C and F, A representative solid follicular cell carcinoma in a 24-month-old *Men1*$^{+/T}$ mouse. Neoplastic cells are in a densely packed nest and form a few minute follicles (*arrows* in F). D–F, Higher magnification of the rectangular regions shown in the *upper panels* (A–C), respectively. Original magnification, ×10.

common feature of the disease seen in 95% of MEN1 patients, which was not detected earlier (14). In addition, there was a very high frequency of gonadal malignancy in our *Men1*$^{+/T}$ mice, including Leydig cell tumors in males and sex-cord stromal cell tumors in females. Although one ovarian tumor was seen in the previous model, no testicular tumors were found (14). The observation that heterozygous *Men1* mutant mice develop gonadal tumors is interesting, because there is so far, to our knowledge, only one case report on the occurrence of a Leydig cell tumor in a MEN1 patient (21). The discrepancy between our observation and those in the previously reported model (14) could have several explanations, including different origins of the ES cells, or the genetic background or the cohort numbers of the mice used in these studies. Nonetheless, the fact that the tumors observed in heterozygous *Men1* mutant mice are mainly located in the endocrine tissues suggests an important physiological role of menin in endocrine organ development.

Consistent with the previous study (14), we found that the tumors of various tissues in our *Men1*$^{+/T}$ mice all showed loss of the wild-type allele of the *Men1* gene. LOH of menin occurs in endocrine tumors of both MEN1 patients and heterozygous *Men1* mutant mice, supporting the notion that the loss of wild-type *Men1* is a prerequisite step toward malignant transformation. However, given the fact that LOH can be detected in both benign and malignant tumors, other genetic events are necessary to fully drive these cells to neoplasia. Because of early embryonic lethality, whether *Men1* null mutation affects normal development or causes tumors in adult endocrine tissues could not be established. Using a conditional knockout strategy, we have recently generated mice with the *Men1* gene specifically disrupted in pancreatic islet β-cells, and these mice develop insulinomas with multiple progression features at an early age (6 months) (22). Overall, these results indicate that the loss of *Men1* is a limiting step in the induction of endocrine cell transformation, although other genetic events may be necessary to complete the process of tumorigenesis.

Despite much effort in genetic and biochemical studies, the biological function of menin remains elusive. We have recently shown that although *Men1* null ES cells and primary embryonic fibroblasts (EFs) proliferate normally *in vitro*, *Men1* null EFs exhibit an early senescence phenotype (15), suggesting that loss of menin may affect telomere-mediated cellular function. In this regard, the *MEN1* product has been localized in telomeres of meiotic cells (23). In addition, menin interacts with a subunit of replication protein A (RPA) (24), suggesting a possible involvement of menin in replication, cell cycle progression and/or cell death. It is thus plausible that the complete loss of menin, *e.g*. via LOH, in adult tissues or specific cell types, such as endocrine cells, would enable cells to accumulate genetic alterations or premalignant events and to acquire a proliferation advantage leading to tumorigenesis.

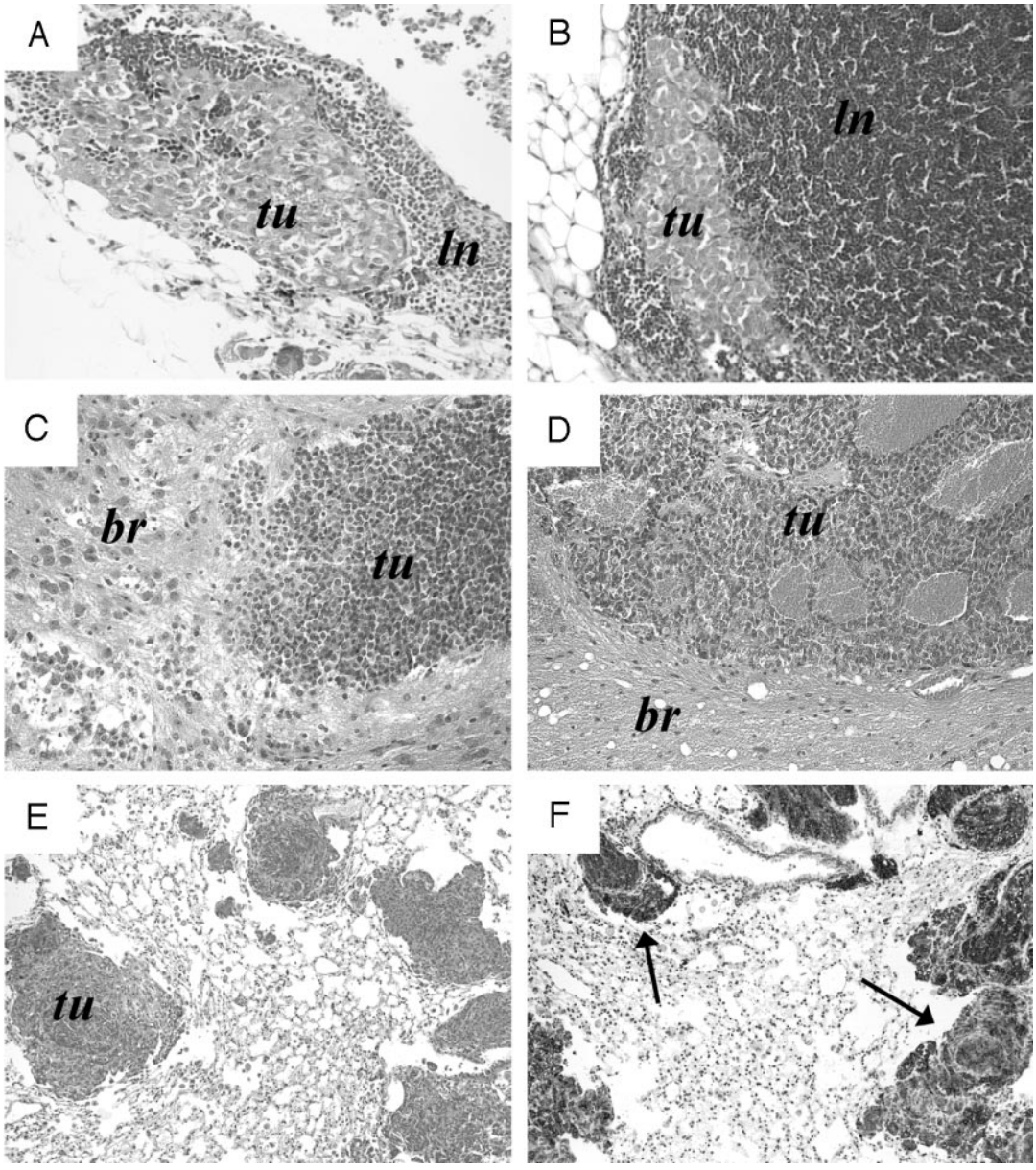
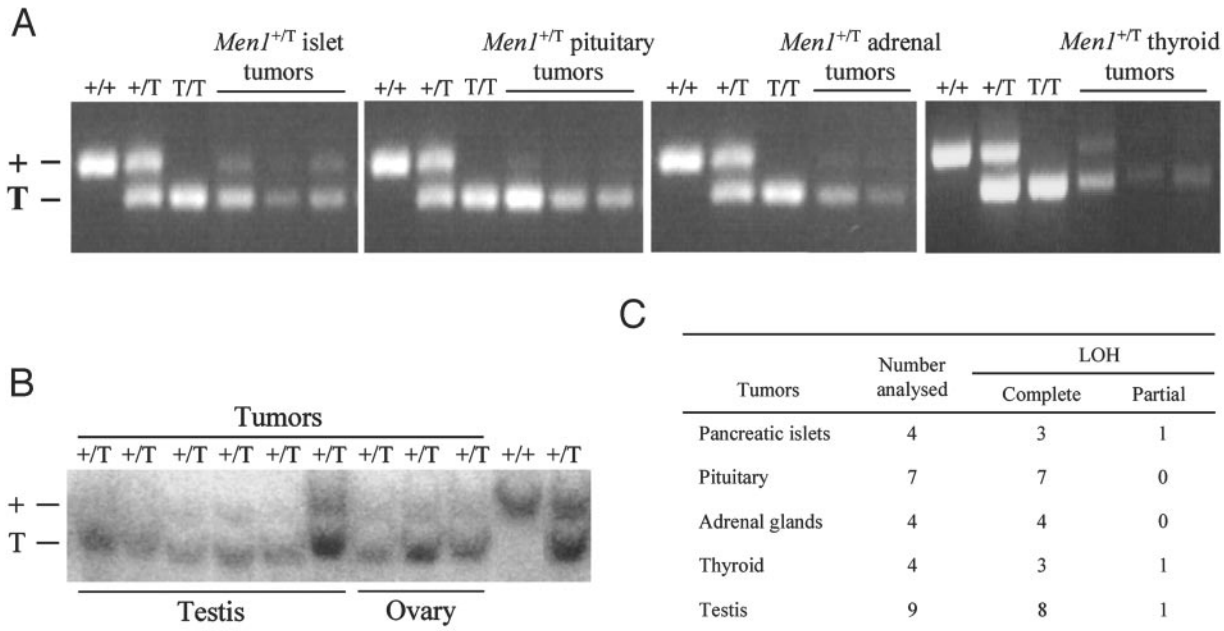


Fig. 8. Metastastic Potential of *Men1*$^{+/T}$ Tumors

A and B, Insulinomas with local lymph node metastasis. *tu*, Insulinoma; *ln*, lymph node. C and D, Prolactinomas invading the brain stem. *tu*, Prolactinomas; *br*, brain. E, Leydig cell tumors being metastasized to the lung, and tumor cells showing positive 3β-HSD immunoreactivity (*arrows* in F). Original magnification of A–D: $\times$20; E and F, $\times$10.

This hypothesis may explain endocrine tumors that arise in MEN1 patients and also in heterozygous *Men1* mutant mice.

In human fetal tissues, menin expression is detectable in the adrenals, heart, kidney, pituitary, testis, thymus, thyroid, and, with the most abundant, in the brain cortex (25). In the mouse, the *Men1* gene is expressed in the entire embryo at E7, and at E13.5, the expression becomes restricted to certain tissues, including the forelimbs, gut, brain, liver, and lung (26, 27). Despite wide expression of the gene, null mutation of *Men1* in mice results in developmental defects in specific organs and heterozygous *Men1* mutant mice develop endocrine tumors (see Refs. 14 and 15), suggesting that other cell- and tissue-specific factors modulate menin's function in specific tissues. In this regard, it is interesting to note that menin has been found to interact with several partners, such as JunD, Smad3, nm23, Pem, and the major components of the nuclear factor-κB family p65, p52, and p50 (28–32). Further studies on the interactions between menin and its partners, especially their physiological and pathological roles in endocrine tissues affected in MEN1 disease, should provide significant clues about the mechanisms of tumorigenesis related to the MEN1 syndrome. Our heterozygous *Men1* mutant mouse



C

Tumors	Number analysed	LOH	
		Complete	Partial
Pancreatic islets	4	3	1
Pituitary	7	7	0
Adrenal glands	4	4	0
Thyroid	4	3	1
Testis	9	8	1
Ovary	6	1	5
Total	34	26	8

Fig. 9. LOH in *Men1*$^{+/T}$ Tumors

A, PCR analysis of the *Men1* allele in the islet, pituitary, adrenal, and thyroid tumors showing partial or complete loss of the wild-type *Men1* (+) allele in comparison with the targeted (T) allele. B, Southern blot analysis of *Men1*$^{+/T}$ tumors showing loss of the wild-type *Men1* (+) allele in tumor samples. C, Summary of LOH analysis in tumors.

model, which faithfully reproduces all the major endocrine tumors seen in MEN1 patients, will be an important tool in such efforts.

MATERIALS AND METHODS

Animals and Genotyping

Mice carrying an inactivated *Men1* allele (*Men1*$^{+/T}$) were generated using a targeting vector, as described previously (15), and were maintained in an 129 background (129/Ola x 129/Sv). Heterozygous *Men1* mutant mice were intercrossed to produce a large cohort of wild-type and *Men1*$^{+/T}$ mice. All animal experiments were conducted in accordance with accepted standards of humane animal care and were approved by the International Agency for Research on Cancer's Animal Care and Use Committee. Genotyping was performed by PCR on DNA extracted from mouse tails with the following two primers: 3f1, 5′-GGATTCTGCCCCAGGC; 3r1, 5′-CACCTCCATCTTACGGTCG. Each PCR was carried out in a 25 μl reaction mixture containing 1 μl of template DNA, 1 μM of each primer, 1 mM of each deoxynucleotide triphosphate and 1 U *Taq* polymerase. The reaction mixture was denatured for 5 min at 94 C and incubated for 35 cycles (denaturing at 94 C for 30 sec, annealing at 56 C for 45 sec, and extension at 72 C for 105 sec).

Histopathological and Immunohistochemical Analyses

Tissues were collected from mice at various time points and fixed in 4% neutral-buffered formaldehyde for at least 24 h, followed by dehydration and paraffin embedding. Histopathological analysis was carried out on 3-μm sections stained with hematoxylin-eosin. Immunohistochemical staining was performed essentially on serial sections, as described previously (33), using antibodies against insulin (polyclonal, 1:400, DAKO, Carpinteria, CA), glucagon (polyclonal, 1:500, NovoCastra, Newcastle upon Tyne, UK), gastrin (polyclonal, 1:200, NovoCastra), human GH (polyclonal, 1: 100, NovoCastra), PRL (monoclonal, 1:200, NovoCastra), and 3β-HSD (polyclonal, 1:2000, gift from Dr. M. Benahmed, Lyon, France).

Quantification of Serum PTH and Insulin Levels

All measurements were carried out on animals between 8 and 26 months of age. Mice were fasted for 4 h before serum collection. Blood was collected from the retro-orbital plexus and serum was obtained after clotting and separation by centrifugation. For PTH, quantification was performed with a mouse PTH ELISA kit (Alpco Diagnostics, Windham, NH). Quantification of serum insulin was performed with a solid-phase two-site ELISA immunoassay, specific for mouse insulin (Ultrasensitive mouse insulin ELISA, Mercodia, Uppsala, Sweden). Assays for each serum sample were performed in duplicate and repeated twice.

LOH Analysis

PCR and Southern blot analyses were performed to check LOH. For PCR analysis, tumor DNA was extracted from microdissected paraffin sections as previously described (33) and amplified using the following three primers: 2f0, 5′-CTTACCTCTTCTCATGTCTG; 2r0, 5′-CTCAGTACATTGCACGGAGA; tk1.1, 5′-GCGTTGCGTGGGGTCAG. Each PCR was

carried out as described under genotyping. Southern blot analysis was performed with a probe of the *Men1* gene, located immediately upstream of exon1, after *Bam*HI and *Bgl*II digestion of DNA extracted either from freshly dissected tumors or several paraffin-embedded tumor sections.

Acknowledgments

We thank Marie-Pierre Cros for the maintenance of the mouse colonies [International Agency for Research on Cancer (IARC), Lyon, France], and Nicole Lyandrat, Sandra Roche and Christine Carreira for histological assistance (IARC). We are also grateful to Dr. M. Benahmed for kindly providing us with the anti-3β-HSD antibody, to Drs. A. Calender and M. Mignon for critical discussion and Dr. J. Cheney for editing the manuscript.

Received April 23, 2003. Accepted June 9, 2003.

Address all correspondence and requests for reprints to: Zhao-Qi Wang, Ph.D., International Agency for Research on Cancer, 150 Cours Albert-Thomas, 69008 Lyon, France. E-mail: zqwang@iarc.fr.

This work was supported by the Association pour la Recherche sur le Cancer, France, the Programme Emergence from the Région Rhône-Alpes, the Ligue contre le Cancer du Rhône, and the Association for International Cancer Research (AICR), UK. P.B. is the recipient of a fellowship from the French Ministry of Education.

REFERENCES

1. Chandrasekharappa SC, Guru SC, Manickam P, Olufemi SE, Collins FS, Emmert-Buck MR, Debelenko LV, Zhuang Z, Lubensky IA, Liotta LA, Crabtree JS, Wang Y, Roe BA, Weisemann J, Boguski MS, Agarwal SK, Kester MB, Kim YS, Heppner C, Dong Q, Spiegel AM, Burns AL, Marx SJ 1997 Positional cloning of the gene for multiple endocrine neoplasia-type 1. Science 276:404–407
2. Lemmens I, Van de Ven WJ, Kas K, Zhang CX, Giraud S, Wautot V, Buisson N, De Witte K, Salandre J, Lenoir G, Pugeat M, Calender A, Parente F, Quincey D, Gaudray P, De Wit MJ, Lips CJ, Hoppener JW, Khodaei S, Grant AL, Weber G, Kytola S, Teh BT, Farnebo FF, Phelan C, Hayward N, Larsson C, Pannet AJ, Forbes S, Bassett JD, Thakker RV 1997 Identification of the multiple endocrine neoplasia type 1 (MEN1) gene. The European Consortium on MEN1. Hum Mol Genet 6:1177–1183
3. Thakker RV 1995 Multiple endocrine neoplasia type 1. In: De Groot LJ, Besser G, Jameson J, Loriaux D, Marshall J, Odell W, Potts J, Rubersteinp A, eds. Endocrinology. Philadelphia: Saunders; 2815–2831
4. Schussheim DH, Skarulis MC, Agarwal SK, Simonds WF, Burns AL, Spiegel AM, Marx SJ 2001 Multiple endocrine neoplasia type 1: new clinical and basic findings. Trends Endocrinol Metab 12:173–178
5. Agarwal SK, Kester MB, Debelenko LV, Heppner C, Emmert-Buck MR, Skarulis MC, Doppman JL, Kim YS, Lubensky IA, Zhuang Z, Green JS, Guru SC, Manickam P, Olufemi SE, Liotta LA, Chandrasekharappa SC, Collins FS, Spiegel AM, Burns AL, Marx SJ 1997 Germline mutations of the MEN1 gene in familial multiple endocrine neoplasia type 1 and related states. Hum Mol Genet 6:1169–1175
6. Giraud S, Zhang CX, Serova-Sinilnikova O, Wautot V, Salandre J, Buisson N, Waterlot C, Bauters C, Porchet N, Aubert JP, Emy P, Cadiot G, Delemer B, Chabre O, Niccoli P, Leprat F, Duron F, Emperauger B, Cougard P, Goudet P, Sarfati E, Riou JP, Guichard S, Rodier M, Meyrier A, Caron P, Vantyghem M, Assayag M, Peix J, Pugeat M, Rohmer V, Vallotton M, Lenoir G, Gaudray P, Proye C, Conte-Devolx B, Chanson P, Shugart Y, Goldgar D, Murat A, Calender A 1998 Germ-line mutation analysis in patients with multiple endocrine neoplasia type 1 and related disorders. Am J Hum Genet 63: 455–467
7. Bassett JH, Forbes SA, Pannett AA, Lloyd SE, Christie PT, Wooding C, Harding B, Besser GM, Edwards CR, Monson JP, Sampson J, Wass JA, Wheeler MH, Thakker RV 1998 Characterization of mutations in patients with multiple endocrine neoplasia type 1. Am J Hum Genet 62:232–244
8. Farnebo F, Teh BT, Kytola S, Svensson A, Phelan C, Sandelin K, Thompson NW, Hoog A, Weber G, Farnebo LO, Larsson C 1998 Alterations of the MEN1 gene in sporadic parathyroid tumors. J Clin Endocrinol Metab 83:2627–2630
9. Zhuang Z, Vortmeyer AO, Pack S, Huang S, Pham TA, Wang C, Park WS, Agarwal SK, Debelenko LV, Kester M, Guru SC, Manickam P, Olufemi SE, Yu F, Heppner C, Crabtree JS, Skarulis MC, Venzon DJ, Emmert-Buck MR, Spiegel AM, Chandrasekharappa SC, Collins FS, Burns AL, Marx SJ, Lubensky IA 1997 Somatic mutations of the MEN1 tumor suppressor gene in sporadic gastrinomas and insulinomas. Cancer Res 57:4682–4686
10. Wautot V, Vercherat C, Lespinasse J, Chambe B, Lenoir GM, Zhang CX, Porchet N, Cordier M, Beroud C, Calender A 2002 Germline mutation profile of MEN1 in multiple endocrine neoplasia type 1: search for correlation between phenotype and the functional domains of the MEN1 protein. Hum Mutat 20:35–47
11. Larsson C, Skogseid B, Oberg K, Nakamura Y, Nordenskjold M 1988 Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. Nature 332:85–87
12. Bystrom C, Larsson C, Blomberg C, Sandelin K, Falkmer U, Skogseid B, Oberg K, Werner S, Nordenskjold M 1990 Localization of the MEN1 gene to a small region within chromosome 11q13 by deletion mapping in tumors. Proc Natl Acad Sci USA 87:1968–1972
13. Debelenko LV, Zhuang Z, Emmert-Buck MR, Chandrasekharappa SC, Manickam P, Guru SC, Marx SJ, Skarulis MC, Spiegel AM, Collins FS, Jensen RT, Liotta LA, Lubensky IA 1997 Allelic deletions on chromosome 11q13 in multiple endocrine neoplasia type 1-associated and sporadic gastrinomas and pancreatic endocrine tumors. Cancer Res 57:2238–2243
14. Crabtree JS, Scacheri PC, Ward JM, Garrett-Beal L, Emmert-Buck MR, Edgemon KA, Lorang D, Libutti SK, Chandrasekharappa SC, Marx SJ, Spiegel AM, Collins FS 2001 A mouse model of multiple endocrine neoplasia, type 1, develops multiple endocrine tumors. Proc Natl Acad Sci USA 98:1118–1123
15. Bertolino P, Radovanovic I, Casse H, Aguzzi A, Wang Z-Q, Zhang CX 2003 Genetic ablation of the tumor suppressor menin causes lethality at mid-gestation with defects in multiple organs. Mech Dev 120:549–560
16. Pipeleers-Marichal M, Somers G, Willems G, Foulis A, Imrie C, Bishop AE, Polak JM, Hacki WH, Stamm B, Heitz PU, Kloppel G 1990 Gastrinomas in the duodenums of patients with multiple endocrine neoplasia type 1 and the Zollinger-Ellison syndrome. N Engl J Med 322:723–727
17. Verges B, Boureille F, Goudet P, Murat A, Beckers A, Sassolas G, Cougard P, Chambe B, Montvernay C, Calender A 2002 Pituitary disease in MEN type 1 (MEN1): data from the France-Belgium MEN1 Multicenter study. J Clin Endocrinol Metab 87:457–465
18. Lubensky IA, Debelenko LV, Zhuang Z, Emmert-Buck MR, Dong Q, Chandrasekharappa S, Guru SC, Manickam P, Olufemi SE, Marx SJ, Spiegel AM, Collins FS, Liotta LA 1996 Allelic deletions on chromosome 11q13 in

multiple tumors from individual MEN1 patients. Cancer Res 56:5272–5278
19. Zedenius J, Wallin G, Svensson A, Grimelius L, Hoog A, Lundell G, Backdahl M, Larsson C 1995 Allelotyping of follicular thyroid tumors. Hum Genet 96:27–32
20. Matsuo K, Tang SH, Fagin JA 1991 Allelotype of human thyroid tumors: loss of chromosome 11q13 sequences in follicular neoplasms. Mol Endocrinol 5:1873–1879
21. Ibarguren RL, Egurrola JA, Echevarria AA, Lafuente JCM, Basante AM, Ortega JLL, Ortuenta IS, Perez MA, Tristan AA, Villalonga MB, Molina FV and Pena CP 1992 Tumor de celulas de Leydig en paciente con neoplasia endocrina multiple tipo I: estudio de un caso y revision de la literatura. Actas Urologias Espanolas 16:650–651
22. Bertolino P, Tong WM, Herrera PL, Casse H, Zhang CX, Wang Z-Q, Pancreatic β-cell-specific ablation of the multiple endocrine neoplasia type 1 (MEN1) gene causes full penetrance of insulinoma development in mice. Cancer Res, in press
23. Suphapeetiporn K, Greally JM, Walpita D, Ashley T, Bale AE 2002 MEN1 tumor-suppressor protein localizes to telomeres during meiosis. Genes Chromosomes Cancer 35:81–85
24. Sukhodolets KE, Hickman AB, Agarwal SK, Sukhodolets MV, Obungu VH, Novotny EA, Crabtree JS, Chandrasekharappa SC, Collins FS, Spiegel AM, Burns AL, Marx SJ 2003 The 32-kilodalton subunit of replication protein A interacts with menin, the product of the MEN1 tumor suppressor gene. Mol Cell Biol 23:493–509
25. Wautot V, Khodaei S, Frappart L, Buisson N, Baro E, Lenoir GM, Calender A, Zhang CX, Weber G 2000 Expression analysis of endogenous menin, the product of the multiple endocrine neoplasia type 1 gene, in cell lines and human tissues. Int J Cancer 85:877–881
26. Stewart C, Parente F, Piehl F, Farnebo F, Quincey D, Silins G, Bergman L, Carle GF, Lemmens I, Grimmond S, Xian CZ, Khodei S, Teh BT, Lagercrantz J, Siggers P, Calender A, Van de Vem V, Kas K, Weber G, Hayward N, Gaudray P, Larsson C 1998 Characterization of the mouse Men1 gene and its expression during development. Oncogene 17:2485–2493
27. Guru SC, Crabtree JS, Brown KD, Dunn KJ, Manickam P, Prasad NB, Wangsa D, Burns AL, Spiegel AM, Marx SJ, Pavan WJ, Collins FS, Chandrasekharappa SC 1999 Isolation, genomic organization, and expression analysis of Men1, the murine homolog of the MEN1 gene. Mamm Genome 10:592–596
28. Agarwal SK, Guru SC, Heppner C, Erdos MR, Collins RM, Park SY, Saggar S, Chandrasekharappa SC, Collins FS, Spiegel AM, Marx SJ, Burns AL 1999 Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription. Cell 96:143–152
29. Kaji H, Canaff L, Lebrun JJ, Goltzman D, Hendy GN 2001 Inactivation of menin, a Smad3-interacting protein, blocks transforming growth factor type beta signaling. Proc Natl Acad Sci USA 98:3837–3842
30. Ohkura N, Kishi M, Tsukada T, Yamaguchi K 2001 Menin, a gene product responsible for multiple endocrine neoplasia type 1, interacts with the putative tumor metastasis suppressor nm23. Biochem Biophys Res Commun 282:1206–1210
31. Lemmens IH, Forsberg L, Pannett AA, Meyen E, Piehl F, Turner JJ, Van de Ven WJ, Thakker RV, Larsson C, Kas K 2001 Menin interacts directly with the homeobox-containing protein Pem. Biochem Biophys Res Commun 286:426–431
32. Heppner C, Bilimoria KY, Agarwal SK, Kester M, Whitty LJ, Guru SC, Chandrasekharappa SC, Collins FS, Spiegel AM, Marx SJ, Burns AL 2001 The tumor suppressor protein menin interacts with NF-κB proteins and inhibits NF-κB-mediated transactivation. Oncogene 20:4917–4925
33. Tong WM, Cortes U, Hande MP, Ohgaki H, Cavalli LR, Lansdorp PM, Haddad BR, Wang ZQ 2002 Synergistic role of Ku80 and poly(ADP-ribose) polymerase in suppressing chromosomal aberrations and liver cancer formation. Cancer Res 62:6990–6996

Addendum

In the article by X. Cui *et al*. (*Mol Endocrinol* **17**:575–588), the following text should have been included in the Acknowledgments section: "This work was supported in part by Research Grants NIH R01CA94118 (to A.V.L.) and USAMRC postdoctoral fellowship DAMD 17-01-1-0133 (to X.C.)." *The authors regret the omission*.