Genetic background influences embryonic lethality and the occurrence of neural tube defects in Men1 null mice: relevance to genetic modifiers

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Abstract

Germline mutations of the multiple endocrine neoplasia type 1 (MEN1) gene cause parathyroid, pancreatic and pituitary tumours in man. MEN1 mutations also cause familial isolated primary hyperparathyroidism (FIHP) and the same MEN1 mutations, in different families, can cause either FIHP or MEN1. This suggests a role for genetic background and modifier genes in altering the expression of a mutation. We investigated the effects of genetic background on the phenotype of embryonic lethality that occurs in a mouse model for MEN1. Men1\(^{+/+}\) mice were backcrossed to generate C57BL/6 and 129S6/SvEv incipient congenic strains, and used to obtain homozygous Men1\(^{++}\) mice. No viable Men1\(^{++}\) mice were obtained. The analysis of 411 live embryos obtained at 9.5–16.5 days post-coitum (dpc) revealed that significant deviations from the expected Mendelian 1:2:1 genotype ratio were first observed at 12.5 and 14.5 dpc in the 129S6/SvEv and C57BL/6 strains respectively (\(P<0.05\)). Moreover, live Men1\(^{++}\) embryos were absent by 13.5 and 15.5 dpc in the 129S6/SvEv and C57BL/6 strains respectively thereby indicating an earlier lethality by 2 days in the 129S6/SvEv strain (\(P<0.01\)). Men1\(^{++}\) embryos had macroscopic haemorrhages, and histology and optical projection tomography revealed them to have internal haemorrhages, myocardial hypotrophy, pericardial effusion, hepatic abnormalities and neural tube defects. The neural tube defects occurred exclusively in 129S6/SvEv embryos (21 vs 0\%, \(P<0.01\)). Thus, our findings demonstrate the importance of genetic background in influencing the phenotypes of embryonic lethality and neural tube defects in Men1\(^{++}\) mice, and implicate a role for genetic modifiers.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder characterised, in man, by the combined occurrence of tumours of the parathyroids, pancreatic islets and anterior pituitary (Thakker 2006). Some patients may also develop adrenal cortical tumours, carcinoid tumours, facial angiofibromas, collagenomas and lipomas. The MEN1 gene, which in man is located on chromosome 11q13 and encodes a 610 amino acid protein (menin), belongs to the class of tumour suppressors (Chandrasekharappa et al. 1997, Lemmens et al. 1997). Menin, which is ubiquitously expressed and is predominantly a nuclear protein, has been shown to interact with a number of proteins that are involved in transcriptional regulation, DNA replication and cell cycle control (Balogh et al. 2006). These interacting proteins include a member of the activating protein-1 (AP-1) transcription factor complex, JunD; placenta and embryonic expression gene product (Pem); mothers against decapentaplegic homolog 3 (Smad3) and major components of nuclear factor-κB (Agarwal et al. 1999, Balogh et al. 2006, Lemos & Thakker 2008). Over 1000 MEN1 germline mutations, which are scattered throughout the \(>9\) kb genomic sequence, have been reported (Lemos & Thakker 2008). The majority (\(>70\%\)) of these mutations are predicted to lead to truncated forms of menin, although there appears to be an absence of genotype–phenotype correlation (Lemos & Thakker 2008). More than 97\% of these MEN1 germline mutations are associated with the multiple development of tumours associated with this syndrome. However, the remaining \(<3\%\) are associated with the sole occurrence of parathyroid tumours, a condition referred to as familial isolated primary hyperparathyroidism (FIHP; Hannan

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et al. 2008, Lemos & Thakker 2008). The 30 MEN1 mutations reported in patients with FIHP are remarkably similar to those reported in patients with MEN1, and 15 (i.e. 50%) of these MEN1 mutations have been reported to occur in both FIHP and MEN1 patients (Table 1; Hannan et al. 2008, Lemos & Thakker 2008). Thus, the same MEN1 mutation can, in different families, cause MEN1 or FIHP and the mechanisms that determine this altered phenotypic expression remain to be elucidated. One mechanism that may influence phenotype modification is the influence of genetic background due to modifier genes (Nadeau 2003a). The effects of such modifier genes and genetic background are difficult to study in man because of the late onset of the disorder and because of the effects of allelic heterogeneity and environmental factors that cannot be readily controlled (Nadeau 2005). However, these variables can be controlled in studies of inbred laboratory mice, which may also develop the disease phenotype within a shorter duration (Nadeau 2001). Indeed, genetic modifiers have been shown, mainly by such studies in mice, to affect penetrance, dominance, modification, expressivity and pleiotrophy (Nadeau 2001, 2003b). We therefore embarked on studying the effects of genetic background on the phenotypic expression of MEN1 in a mouse model that was generated by deleting exons 1 and 2 (Fig. 1A), which contains the ATG translation start signal (Lemos et al. 2007). Mice deleted for one allele, i.e. heterozygotes (Men1+/−) develop parathyroid, pancreatic islet cell, anterior pituitary and adrenal cortical tumours after the age of 12 months (Lemos et al. 2007), and this is similar to that reported in other MEN1 mouse models (Crabtree et al. 2001, Bertolino et al. 2003b, Loffler et al. 2007). Moreover, mice deleted for both alleles, i.e. homozygotes (Men1−/−),

Table 1

<table>
<thead>
<tr>
<th>Exon</th>
<th>Codon</th>
<th>Predicted effect</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>83–84</td>
<td>Frameshift</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>363</td>
<td>In-frame deletion of Glu363</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>559</td>
<td>Frameshift</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>3</td>
<td>153</td>
<td>Asp153Val</td>
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<td>4</td>
<td>225</td>
<td>Gly225Arg</td>
</tr>
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<td>7</td>
<td>4</td>
<td>255</td>
<td>Glu255Lys</td>
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<td>260</td>
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<td>10</td>
<td>527</td>
<td>Stop signal at codon 527</td>
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<td>11</td>
<td>Boundary between exon 3 and intron 3</td>
<td>218</td>
<td>Donor splice-site alteration</td>
</tr>
<tr>
<td>12</td>
<td>Boundary between intron 4 and exon 5</td>
<td>Nucleotide 784−9d</td>
<td>Acceptor splice-site alteration</td>
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<td>13</td>
<td>Boundary between exon 5 and intron 5</td>
<td>Nucleotide 824+1d</td>
<td>Donor splice-site alteration</td>
</tr>
<tr>
<td>14</td>
<td>Boundary between exon 9 and intron 9</td>
<td>Nucleotide 1350+1d</td>
<td>Donor splice-site alteration</td>
</tr>
<tr>
<td>15</td>
<td>Gross deletion</td>
<td>Gross deletion</td>
<td>Cavaco et al. (2002)b and Cebrian et al. (2003)f</td>
</tr>
</tbody>
</table>

aMutation number.
bReference describing presence of mutation in MEN1 patient.
cReference describing presence of mutation in FIHP patient.
dNucleotide positions start from the initiation codon and are based on the cDNA reference sequence (GenBank accession number NM_130799.1).
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**Materials and Methods**

**Generation of Men1<sup>+/−</sup> mice incipient congenic strains**

A mouse model for MEN1 (Lemos et al. 2007) that had a targeted disruption of exons 1 and 2, which contains the translation initiation codon ATG, was used (Fig. 1A). The loss of the initiation codon by the targeted disruption would result in a loss of menin expression, and hence have similarities to the situation in >70% of MEN1 patients who have mutations that are predicted to lead to truncated forms of menin (Lemos & Thakker 2008). Men1<sup>+/−</sup> mice have been reported to develop parathyroid, pancreatic islet, anterior pituitary, adrenal cortical and gonadal tumours (Lemos et al. 2007). F1 Men1<sup>+/−</sup> mice were mated to C57BL/6J and 129S6/SvEvTac inbred mice to initiate two lines of Men1 knockout mice on different genetic backgrounds. Heterozygotes were consecutively backcrossed to each of these strains for five to nine generations, to generate two different incipient congenic strains with 96.9–99.8% genetic identity in either the C57BL/6 or the 129S6/SvEv inbred strains. Following this, heterozygote intercrosses were made to obtain homozygote mice. Mice were kept in accordance with UK Home Office welfare guidelines and project license restrictions.

**Genotyping of mice**

Genotypes of adult, neonatal and embryonic mice were determined by PCR analysis using DNA extracted from appropriate tissues (e.g. tails, auricular biopsies or amniotic sacs) and Men1 gene-specific primers, as reported (Lemos et al. 2007). Primers 1R (5'-CCA AAC TCC ATG TTC-3') and 1F (5'-CAC GAA GTC TGT CAG TAT GAC CCT GTT TCC-3') yielded a 735 bp wild-type fragment and primers 1R and NeoF, which were specific for the Neomycin transferase gene, yielded a 499 bp product from the mutant (M) allele. (B) PCR analysis for the genotyping of embryos was undertaken using PCR and Men1-specific primers (1R and 1F, or 1R and NeoF). A standard marker (m), as a 100 bp ladder is shown. (C) Western blot analysis for menin expression in 11.5 dpc Men1<sup>+/−</sup>, Men1<sup>++/−/−</sup> and Men1<sup>−/−</sup> embryos demonstrating menin expression in Men1<sup>++/−</sup> and Men1<sup>−/−</sup> embryos, but its absence in Men1<sup>−/−</sup> embryos. An anti-α-tubulin antibody was used as a control.

are embryonically lethal with abnormalities of the heart, liver, neural tube and cranio-facial bones (Crabtree et al. 2001, Bertolino et al. 2003a). Because of the late onset (over 12 months of age) of tumours in Men1<sup>+/−</sup> mice, we decided to study the possible effects of genetic modifiers and background on the phenotype of embryonic lethality in Men1<sup>−/−</sup> mice, as this would facilitate establishing the proof of principle of phenotypic modification by genetic background in MEN1. In order to pursue these studies, we established Men1<sup>+/−</sup> mice on two different genetic backgrounds that consisted of the C57BL/6 and 129S6/SvEv strains.

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**Figure 1** Genotyping and menin expression in embryos. Embryos were obtained from Men1<sup>+/−</sup> intercrosses. (A) Men1<sup>+/−</sup> mice had one target allele in which exons 1 and 2 of the Men1 gene had been replaced by a PGK-Neo<sup>R</sup> cassette (Lemos et al. 2007). This targeted disruption would result in a loss of the initiation codon, ATG, in exon 2 and hence a loss of menin expression, which is similar to the situation in man where the majority (>70%) of patients have mutations that lead to truncated forms of menin. The location of the epitope (amino acids 571–611, in exon 10) against which the rabbit polyclonal anti-menin antibody has been raised is indicated by the solid horizontal black bar with an asterisk. Thus, use of this antibody would detect only full-length menin, but not the truncated forms. Primers 1R and 1F, which were located in the 5'-UTR of the Men1 gene, yielded a 735 bp product from the wild-type (WT) allele. Use of primers 1R and NeoF, which were specific for the Neomycin transferase gene, yielded a 499 bp product from the mutant (M) allele. (B) PCR analysis for the genotyping of embryos was undertaken using PCR and Men1-specific primers (1R and 1F, or 1R and NeoF). A standard marker (m), as a 100 bp ladder is shown. (C) Western blot analysis for menin expression in 11.5 dpc Men1<sup>+/−</sup>, Men1<sup>++/−/−</sup> and Men1<sup>−/−</sup> embryos demonstrating menin expression in Men1<sup>++/−</sup> and Men1<sup>−/−</sup> embryos, but its absence in Men1<sup>−/−</sup> embryos. An anti-α-tubulin antibody was used as a control.

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Phenotype studies of embryos

Embryos from Men1+/− intercrosses were removed by Caesarean section at successive days post-coitum following timed matings and the ages of the embryos were confirmed by assessing the development of external structures using the Theiler staging system (Theiler 1989; available online at http://genex.hgu.mrc.ac.uk/Atlas/Theiler_book_download.html). Amniotic sacs were collected for genotyping using PCR as described above. Embryos were inspected for overall external morphology, photographed and fixed in 4% paraformaldehyde. In order to characterise internal abnormalities, three-dimensional imaging by optical projection tomography (OPT) of the embryos was undertaken, using previously described methods (Sharpe et al. 2002). OPT is a technique that allows three-dimensional imaging of biological specimens and the analysis of virtual sections in any orientation. Thus, OPT has several advantages over other imaging techniques such as confocal microscopy, optical coherence tomography, microscopic magnetic resonance imaging or reconstruction of serial sections (Sharpe et al. 2002, Sharpe 2003). A total of 25 embryos (5 Men1+/−, 4 Men1+/− and 16 Men1−/−) aged 10.5–13.5 dpc were analysed by OPT; 16 of the embryos were on a C57BL/6 background (four Men1+/+, two Men1+/− and ten Men1−/−) and nine were on a 129S6/SvEv background (one Men1+/+, two Men1+/− and six Men1−/−). For histological examination, embryos were embedded in paraffin, and transverse sections (5–7 μm thickness) were obtained and stained with haematoxylin and eosin (Wang et al. 2008). Immunostaining for menin expression was performed using a menin antibody (rabbit anti-menin, Abcam), as follows. Wax sections for immunohistochemistry were transferred to electrostatically charged slides (Superfrost Plus, Menzel Glaser, Leuven, Belgium), de-paraffinised in xylene substitute and rehydrated via graded ethanol. Antigen retrieval was performed in citrate buffer, pH 6, in an autoclave, followed by treatment with 3% aqueous hydrogen peroxide for 30 min. After blocking with serum for 30 min, sections were incubated with menin antibody (1:500) at 4 °C, overnight. HRP-labelled goat anti-rabbit polymer system (EnVision + System-HRP (DAB), Dako UK Ltd, Ely, UK) was used to develop the specific signals.

Statistical analysis

The proportion of live Men1−/− embryos at each gestational age was compared with the theoretically expected Mendelian proportion (25%) by performing the χ² goodness-of-fit test (χ²G.O.F.) with one degree of freedom and the results expressed as two-tailed P values. The proportion of live Men1−/− embryos at each age was also compared between the two different genetic backgrounds by performing Fisher’s exact test, and the threshold for statistical significance was set at P<0.05.

Results

Men1+/− mice, established on the C57BL/6 or 129S6/SvEv strains, were intercrossed. The genotypes of the offspring (Table 2; newborn and embryos) were determined by the use of PCR and Men1-specific primers (Fig. 1B) and the expression of menin in Men1+/+ and Men1+/−, and its absence in Men1−/− embryos demonstrated by western blot analysis (Fig. 1C). The absence of Men1−/− mice among 44 newborn offspring, demonstrated that Men1−/− mice from this model for MEN1, generated by a targeted disruption of exons 1 and 2 (Fig. 1A), are embryonically lethal. This finding of embryonic lethality is similar to that previously published from other MEN1 mouse models, which have reported that lethality commences at about 12.5 dpc (Crabtree et al. 2001, Bertolino et al. 2003a). Men1+/− embryos were viable without an increased mortality and had normal development.

Table 2 Multiple endocrine neoplasia type 1 (Men1) genotype analysis of 499 mice obtained from Men1+/− intercrosses on two different genetic backgrounds. A χ² goodness-of-fit test with one degree of freedom was used to compare the observed proportions of live homozygous mutant offspring (−/−) with the expected Mendelian proportion (25%) and the results were expressed as two-tailed P values. Significant deviations from the expected frequency of live Men1−/− embryos were observed from 12.5 days post-coitus (dpc) on the 129S6/SvEv background and from day 14.5 dpc on the C57BL/6 background.

<table>
<thead>
<tr>
<th>Age (dpc)</th>
<th>C57BL/6 No. of live (dead) offspring</th>
<th>129S6/SvEv No. of live (dead) offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men1+/+</td>
<td>Men1+/−</td>
</tr>
<tr>
<td>9–10</td>
<td>3 (0)</td>
<td>2 (0)</td>
</tr>
<tr>
<td>11–12</td>
<td>1 (0)</td>
<td>6 (0)</td>
</tr>
<tr>
<td>13–14</td>
<td>14 (0)</td>
<td>25 (0)</td>
</tr>
<tr>
<td>15–16</td>
<td>16 (1)</td>
<td>35 (0)</td>
</tr>
<tr>
<td>17–18</td>
<td>13 (0)</td>
<td>15 (1)</td>
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<tr>
<td>19–20</td>
<td>8 (0)</td>
<td>12 (0)</td>
</tr>
<tr>
<td>21–22</td>
<td>4 (0)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>Newborn</td>
<td>7 (0)</td>
<td>13 (0)</td>
</tr>
</tbody>
</table>

NS, not significant.
indicating that haploinsufficiency of Men1 is unlikely to affect normal embryogenesis. In order to search for the effects of genetic background on the embryonic lethality, analysis of embryos (total=499 embryos; 411 alive and 88 dead) at different stages of development (9.5–16.5 dpc), from the two different incoptic congenic strains was undertaken (Table 2). All Men1<sup>−/−</sup> embryos were alive up to 10.5 dpc in both strains and the expected Mendelian ratio of 1:2:1 for Men1<sup>+/+</sup>:Men1<sup>+/−</sup>:Men1<sup>−/−</sup> for live embryos obtained from Men1<sup>+/−</sup> intercrosses was observed (Table 2). After 10.5 dpc, dead Men1<sup>−/−</sup> embryos were observed with increasing frequency such that at later gestational stages (12.5 dpc in the 129S6/SvEv strain and 14.5 dpc in the C57BL/6 strain), there was a significant deviation from the expected Mendelian ratio, which would result in 25% of Men1<sup>−/−</sup> live embryos (Table 2 and Fig. 2). In addition, survival of Men1<sup>−/−</sup> embryos was significantly reduced by 2 days in the 129S6/SvEv strain when compared with those in the C57BL/6 strain (Table 2 and Fig. 2). These results demonstrate that genetic background, and hence likely genetic modifiers, influences the phenotype of embryonic lethality in Men1<sup>−/−</sup> mice.

The causes of the embryonic lethality in both strains were investigated by macroscopic examination for external abnormalities, OPT for internal abnormalities, and by histology (Table 3). Macroscopic examination in 58 live Men1<sup>−/−</sup> embryos (39 on the C57BL/6 and 19 on the 129S6/SvEv strain) revealed that 67% (n=39) did not have external abnormalities and that only 33% (n=19) had abnormalities that included delayed development (DD), haemorrhages (HA), neural tube defects (NT) in the form of exencephaly and abnormal fluid accumulation (oedema, OE) along the dorsal region (Fig. 3); among the Men1<sup>−/−</sup> embryos on the C57BL/6 background, 23% (n=9) had such external abnormalities, while among those on the 129S6/SvEv background, 52% (n=10) had these external abnormalities (Table 3). The Men1<sup>−/−</sup> embryos also had internal haemorrhages (Fig. 3G–I). In order to investigate further for internal structural abnormalities that may contribute to embryonic lethality phenotype, Men1<sup>−/−</sup>, Men1<sup>+/−</sup> and control Men1<sup>+/+</sup> embryos, aged from 10.5 to 13.5 dpc, were studied by OPT. The 16 Men1<sup>−/−</sup> embryos included those that were externally normal (n=6, three on the C57BL/6 and three on the 129S6/SvEv background) and those with visible malformations (n=10, seven on the C57BL/6 and three on the 129S6/SvEv background) and ranged in age from 10.5 to 13.5 dpc. Eleven of the 16 Men1<sup>−/−</sup> embryos (nine on the C57BL/6 and two on the 129S6/SvEv background) had myocardial hypotrophy (MH), pericardial effusion (PE) and a reduction in liver size (LS); two Men1<sup>−/−</sup> embryos (both on the 129S6/SvEv background) had MH and PE; one Men1<sup>−/−</sup> embryo, which was on the 129S6/SvEv background, had only LS and two Men1<sup>−/−</sup> embryos had none of these internal abnormalities, one of these being on the C57BL/6 with OE, and the other being on the 129S6/SvEv background with a NT. Eight of the ten Men1<sup>−/−</sup> embryos with external abnormalities also had internal abnormalities and these occurred with the following associations: DD occurred in with MH, PE and LS in four Men1<sup>−/−</sup> embryos on the C57BL/6 background, one of which also had HA, and in association with MH and PE in one Men1<sup>−/−</sup> embryo on the 129S6/SvEv background; OE

### Table 3 Abnormalities in Men1<sup>−/−</sup> live embryos on the C57BL/6 (n=39) and 129S6/SvEv (n=19) backgrounds

<table>
<thead>
<tr>
<th>Abnormalities</th>
<th>C57BL/6&lt;sup&gt;a&lt;/sup&gt;</th>
<th>129S6/SvEv&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Developmental delay</td>
<td>+ (4/39)</td>
<td>+ (2/19)</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>+ (1/39)</td>
<td>+ (1/19)*</td>
</tr>
<tr>
<td>Myocardial hypotrophy</td>
<td>+ (9/10)</td>
<td>+ (4/6)</td>
</tr>
<tr>
<td>Pericardial effusion</td>
<td>+ (9/10)</td>
<td>+ (4/6)</td>
</tr>
<tr>
<td>Oedema</td>
<td>+ (4/39)</td>
<td>− (0/19)&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver size decreased</td>
<td>+ (9/10)</td>
<td>+ (3/6)</td>
</tr>
<tr>
<td>Neural tube defect</td>
<td>− (0/39)</td>
<td>+ (4/19)&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>Embryonic lethality by dpc</td>
<td>14:5</td>
<td>12:5</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.05, †P<0.01; Fisher’s exact test.

<sup>a</sup>+, Presence; −, absence of abnormality. Men1<sup>−/−</sup> embryos with the abnormality are represented as the numerator and the total of Men1<sup>−/−</sup> embryos examined are represented as the denominator in the fractions shown in parenthesis.

<sup>a</sup>Based on OPT analysis of a subset of 16 Men1<sup>−/−</sup> embryos (ten on the C57BL/6 and six on the 129S6/SvEv background).

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Figure 2 Survival of Men1<sup>−/−</sup> embryos on two different genetic backgrounds. The proportion of Men1<sup>−/−</sup> live embryos from the total number (i.e., Men1<sup>+/+</sup> + Men1<sup>+/−</sup> + Men1<sup>−/−</sup>) at each gestational stage is shown. The expected Mendelian ratio from the Men1<sup>+/−</sup> intercrosses of Men1<sup>+/−</sup>:Men1<sup>+/−</sup>:Men1<sup>−/−</sup> offspring is 1:2:1 yielding an expected proportion of 25% for Men1<sup>−/−</sup> embryos. In 129S6/SvEv Men1 mice, this expected proportion was observed at 10.5 and 11.5 dpc, but was significantly lower, at 6:5%, by ≥12.5 dpc. In contrast, in the C57BL/6 strain, the expected proportion was observed at 10.5, 11.5 and 12.5 dpc, but was significantly lower at 15:2%, by ≥13.5 dpc. Furthermore, there were no surviving Men1<sup>−/−</sup> embryos at 13.5 dpc in the 129S6/SvEv strain and at 15.5 dpc in the C57BL/6 strain. A comparison of the survival of the Men1<sup>−/−</sup> embryos in the two strains revealed that, at 12.5 and 13.5 dpc, significantly fewer Men1<sup>−/−</sup> embryos survived in the 129S6/SvEv strain (*P=0.031, **P=0.004, Fisher’s exact test).
occurred with MH, PE and LS in two Men1\(^{-/-}\) embryos on the C57BL/6 background; NT occurred with MH, PE and LS in one Men1\(^{-/-}\) embryo on the 129S6/SvEv background. All Men1\(^{-/-}\) embryos with normal external morphology (three on the C57BL/6 and three on the 129S6/SvEv background) were found, by OPT, to have internal structural abnormalities that included MH, PE and LS (Fig. 4); all the three Men1\(^{-/-}\) embryos on the C57BL/6 background had MH, PE and LS; and among the three Men1\(^{-/-}\) embryos on the 129S6/SvEv background, one had MH, PE and LS, one had MH and PE, and one had LS only. These abnormalities were confirmed by histological analysis of transverse sections obtained from the same embryos that had been studied by OPT. These histology studies revealed a thin myocardium with sparse trabeculation and also a reduced cell density in the liver with the presence of large intercellular spaces (Fig. 5). Immunostaining using an anti-menin antibody demonstrated menin expression in the nuclei of the cardiac cells and hepatocytes of the Men1\(^{+/+}\) embryos, but a loss of menin expression in the Men1\(^{-/-}\) embryos (Fig. 5).

These cardiac and hepatic abnormalities (Figs 4 and 5) have similarities to those reported to occur in embryos lacking the AP-1 family member c-Jun (Hilberg et al. 1993, Eferl et al. 1999). Moreover, menin has been reported to interact with another AP-1 family member, JunD (Agarwal et al. 1999), and homozygous deletion of JunD results in apparently normal mouse embryos, with only adult mutant males showing mild phenotypic features that consisted of impaired growth, hormone imbalance and age-dependent defects in reproduction due to impaired spermatogenesis (Jochum et al. 2001). We therefore investigated different members of the AP-1 family, such as c-Jun, JunD and JunB, for altered expression by using western blot analysis and protein extracts obtained from Men1\(^{+/+}\), Men1\(^{+/+}\) and Men1\(^{-/-}\) 11.5 dpc embryos from

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Figure 3 Embryos obtained from Men1\(^{+/+}\) intercrosses. Embryos obtained (A–C) on the 129S6/SvEv and (D–F) C57BL/6 backgrounds, and at the different gestational ages of (A and B) 10.5 dpc, (C) 11.5 dpc, (D) 12.5 dpc and (E and F) 13.5 dpc, are shown. Men1\(^{+/+}\) and Men1\(^{-/-}\) embryos appeared normal and were similar in size at all gestational ages in both the 129S6/SvEv and C57BL/6 strains. Men1\(^{-/-}\) embryos on the 129S6/SvEv background were normal up to 9.5 dpc but were smaller, presumably due to developmental delay, and showed abnormalities from 10.5 dpc, which were associated with death by 13.5 dpc (Fig. 2). Men1\(^{+/+}\) embryos on the C57BL/6 background were normal up to 11.5 dpc, but showed abnormalities from 12.5 dpc, which were associated with death by 15.5 dpc (Fig. 2). The range of abnormalities in the Men1\(^{-/-}\) embryos is represented: (A) macroscopic haemorrhages (arrow) in thoraco-abdominal region; (B) developmental delay; (C) exencephaly and developmental delay; (D) absence of external malformations; (E) oedema with fluid accumulation along the dorsal region; and (F) macroscopic haemorrhages (arrows) in the head, thoracic and abdominal regions. (A–F) Scale bars (1 mm).

(G–I) Histological analysis using H&E sections obtained from the 13.5 dpc Men1\(^{-/-}\) embryo, which is shown on the right hand panel of (F), and is on the C57BL/6 background, to illustrate internal haemorrhages in the (G) heart, (H) liver and (I) central nervous system (CNS). (G–I) Scale bars (0.1 mm).
the 129S6/SvEv and C57BL/6 backgrounds. The expression of c-Jun, JunD and JunB in the Men1
K/K embryos was similar to that observed in the Men1 C/C and Men1 C/C embryos (data not shown), thereby indicating that the cardiac and hepatic abnormalities observed in the Men1/K/K embryos (Figs 4 and 5) are likely to arise without involvement of these AP-1 family members.

The abnormalities observed in the Men1−/− embryos (Figs 3 and 5 and Table 3) from our Men1 model are similar to those reported in other MEN1 mouse models, in which Men1−/− mice died between 11.5 and 13.5 dpc with growth retardation and defects in the nervous system, heart and liver (Crabtree et al. 2001, Bertolino et al. 2003a). However, these other studies investigated Men1−/− embryos on a 129/Sv or mixed background and therefore could not assess the effects of genetic background. Our studies show that although some of the structural abnormalities (e.g. DD) are similar in the 129S6/SvEv and C57BL/6 genetic backgrounds (Table 3), there are nevertheless important differences; these relate to the neural tube defects (Fig. 3C) and OE (Fig. 3E). The neural tube defects were found only in 129S6/SvEv Men1−/− embryos and not in C57BL/6 Men1−/− embryos (21 vs 0%; Fisher’s exact test P=0.009; Table 3). OE with fluid accumulation along the dorsal region (Fig. 3E) was only found in Men1−/− embryos aged 13.5 dpc or older and hence this was limited to the C57BL/6 strain, as 129S6/SvEv embryos did not survive beyond 12.5 dpc. These results are consistent with a role for genetic background, and hence likely genetic modifiers, influencing neural tube development in Men1−/− mice.

Figure 4 Optical projection tomography (OPT) scans obtained from Men1+/+ and Men1−/− embryos on the C57BL/6 background at 12.5 dpc. In the Men1−/− embryo, a markedly smaller liver and pericardial effusion (arrowheads, B) were observed. Transverse thoracic sections confirmed the occurrence of the pericardial effusion in the Men1−/− embryos and also showed a reduced thickness of the myocardium (arrowheads, D). LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium; Li, liver; St, stomach.

Figure 5 Pathological changes detected by H&E and menin immunostaining of sections of the heart and liver. Men1+/+ and Men1−/− embryos, on the C57BL/6 background, at 12.5 dpc were studied. (A) Transverse section from Men1+/+ embryo revealed a normal left ventricular morphology but (B) in an Men1−/− embryo, the ventricular wall is significantly thinner and the density of the trabeculae is lower. (C) Men1+/+ embryos expressed menin in the nuclei of myocardial cells, whereas (D) Men−/− embryos had a loss of menin expression in all cells. (E) Transverse section from Men1+/+ embryo revealed normal hepatic architecture, but (F) in Men1−/− embryos, the liver had decreased cellularity with the presence of large intercellular spaces. (G) Men1+/+ embryos expressed menin in the nuclei of hepatocytes, whereas (H) Men1−/− embryos had a loss of menin expression in all cells. dv, ductus venosus. Scale bar=0.1 mm.
Discussion

Our results, which demonstrate that genetic background is an important determinant of the phenotypes of the stage of embryonic lethality (Table 2 and Fig. 2) and of neural tube defects (Fig. 3) in Men1−/− mouse embryos, are consistent with a role for genetic modifiers influencing the expression of the MEN1 phenotype. Thus, these results help to establish the proof of principle of phenotypic modification by genetic background in MEN1. The influence of genetic background on neural tube closure defects resulting from the targeted disruption of other genes (e.g. p53, Cart1, p300, Casp3, Ski and Nog) has been previously reported and the decreased frequency of neural tube defects on the C57 background when compared with the 129-related background observed (Armstrong et al. 1995, Sah et al. 1995, Zhao et al. 1996, Yao et al. 1998, Colmenares et al. 2002, Leonard et al. 2002, Stottmann et al. 2006, Harris & Juriloff 2007). Thus, our findings are in agreement with these previous reports and support the occurrence of different genetic modifiers between the C57 and 129-related strains.

The observed reduction in LS in the Men1−/− embryos (Fig. 4) in our study differs from the reported absence of hepatic abnormalities in adult conditional Men1 knockout mice that had a homozygous deletion of the Men1 gene in the liver (Scacheri et al. 2004). One possible explanation is that the loss of both Men1 alleles in the Men1−/− embryos may result in metabolic abnormalities such as hypoxia, which may be secondary to the observed cardiac abnormalities of MH and PE (Figs 4 and 5), and that such extra-hepatic metabolic abnormalities may contribute to the decrease in LS. Likewise, the cardiac abnormalities of MH and PE may be secondary to hypoxia or other such metabolic alterations that may primarily arise at extra-cardiac sites.

The finding of PE (Fig. 4) in the Men1−/− embryos has not been previously reported. It seems likely that the MH (Fig. 5) is contributing to cardiac failure and the observed OE (Fig. 3), which has previously been reported (Crabtree et al. 2001, Bertolino et al. 2003a), and to the PE (Fig. 4). It is likely that the other Men1−/− mice from the other models also develop PE, but this may not have been detected by the histological methods; this illustrates the value of OPT in revealing additional aspects of mutant morphology. Indeed, this is similar to the situation reported in the asplenic phenotype of Bapx1 null mice in which OPT was able to reveal the presence of abnormal ventral surfaces of grooves and ridges in mutant stomachs that may have been missed because of artefacts induced by mounting the sections onto glass slides (Sharpe et al. 2002). The application of OPT to study endocrine glands in embryos and adults promises to be useful, particularly as it can enable three-dimensional imaging to be undertaken, as is illustrated by a recent study of intact adult mouse pancreas that performed cellular and molecular assessments to investigate autoimmune insulitis in type 1 diabetes mellitus (Alenantalo et al. 2008).

In summary, our study, which has demonstrated the effects of genetic background on the phenotypes of embryonic lethality and neural tube defects in Men1−/− mice, helps to establish the proof of principle that genetic modifiers may influence the expression of the Men1−/− mutation in mice. This has relevance for studies in mice and man, as it opens the way for longer-term studies aimed at elucidating the basis of endocrine tumour development in different glands, and the mechanisms determining the development of FIHP and MEN1 in patients with MEN1 mutations. Indeed, modifier genes discovered in mice have often been relevant to the study of human diseases, such as cystic fibrosis, and familial adenomatous polyposis (Nadeau 2001, 2003b). Mouse embryonic lethality associated with null mutations of several genes has been shown to be strain dependent and this has allowed the mapping of modifier loci, as was the case for the Tgß1 and p53 knockout mice (Bonyadi et al. 1997, Evans et al. 2004). Genetic modifiers of Men1 remain to be identified but their identification could provide a better understanding of the function of menin and its molecular interactions in mammalian development and in endocrine tumourigenesis.

Declaration of interest

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

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References

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