Deregulation of anti-Mullerian hormone/BMP and transforming growth factor-β pathways in Leydig cell lesions developed in male heterozygous multiple endocrine neoplasia type 1 mutant mice

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Abstract

Multiple endocrine neoplasia type 1 (MEN1) results from the mutation of the predisposing gene, MEN1. Heterozygous Men1 mutant mice previously generated by several laboratories, including ours, mimic largely MEN1 pathology. Interestingly, our heterozygous Men1 mutant mice exhibit not only the endocrine tumours commonly seen in MEN1 patients, but also Leydig cell tumours (LCT) with high frequency, accompanied systematically by loss of the wild-type Men1 allele. As there exists a similarity of tumour phenotype between these mice and those mutated for the components of anti-Mullerian hormone (AMH)/bone morphogenic protein (BMP) pathway belonging to transforming growth factor-β (TGF-β) family, we investigated the expression and the activity of this pathway, known to have an important biological role in Leydig cells. Here, we report that the expression of AMH receptor type 2 is reduced in Men1 LCTs. Both immunostaining and western blot analyses also demonstrate a markedly decreased nuclear expression of Smad1, 3, 4 and 5 in the tumours. More interestingly, we show that the reconstituted menin expression in Men1-deficient Leydig cells derived from LCTs can significantly increase the transcriptional activity of a BMP pathway target promoter, XVent2. Furthermore, we found that the expression of p18, p27 and cyclin dependant kinase 4 (Cdk4), targets of TGF-β pathways, is altered in the Leydig cell lesions. Our data provide the evidence of the deregulation of AMH/BMP and TGF-β pathways in mouse Men1 LCTs, highlighting their involvement in tumorigenesis of Leydig cells due to Men1 inactivation.

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Introduction

The patients with multiple endocrine neoplasia type 1 (MEN1), a hereditary syndrome transmitted with an autosomal dominant trait, predispose to the occurrence of multiple endocrine tumours of the parathyroids, pancreas, anterior pituitary and adrenal cortex (MEN1, OMIM 131100). Other endocrine and non-endocrine tumours, such as forcut carcinoids, follicular thyroid tumours, angiofibroma, lipoma and smooth muscle

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tumours, can also be associated with the disease (Thakker 1995). Germ line mutations in the MEN1 gene have been detected in the majority of familial MEN1 cases (Agarwal et al. 1997, Bassett et al. 1998, Giraud et al. 1998), and somatic mutations have also been found in several types of sporadic endocrine tumour, especially sporadic parathyroid adenomas (Farnebo et al. 1998), gastrinomas and insulinomas (Zhuang et al. 1997). Both germ line and sporadic mutations show a typical loss of function profile, with different types of mutation detected along the whole coding sequence (Schussheim et al. 2001, Wautot et al. 2002). However, these observations do not establish any genotype–phenotype correlation. The loss of heterozygosity frequently observed in MEN1 tumours supports the hypothesis that the MEN1 gene acts as a tumour suppressor in affected cells (Larsson et al. 1988, Bystrom et al. 1990, Debelenko et al. 1997).

In order to study the mechanisms involved in multiple endocrinopathy related to MEN1 gene inactivation, several laboratories, including ours, have generated Men1 mutant mice (Crabtree et al. 2001, Bertolino et al. 2003a,b, Biondi et al. 2004). The heterozygous Men1 mutant mice develop, starting at around 12 months of age, multiple endocrine tumours. To our surprise, in addition to the endocrine tumours similar to those commonly described in MEN1 patients, we observed gonadal sex-cord stromal tumours with high frequency in our heterozygous Men1 mutant mice, including Leydig cell tumours (LCTs) in males and sex-cord stromal cell tumours of the ovary in females (Bertolino et al. 2003a). Interestingly, Loffler et al. (2007) have recently also reported the occurrence of gonadal sex-cord stromal tumours in their heterozygous Men1 mutant mice. In our hands, the loss of heterozygosity (LOH) were systematically detected in these tumours, suggesting a direct link between Men1 gene inactivation and the development of these tumours in Men1<sup>+/<i>T</i></sup> mice (Bertolino et al. 2003a, Hussein et al. 2007). However, although a case of LCT has been reported in a MEN1 patient (Ibarguren et al. 1992), the clinical significance of this observation in mouse Men1 model remains to be elucidated.

Interestingly, we have noticed that several mouse models with deficiency of transforming growth factor-β (TGF-β) family components present similar tumour phenotypes to the above-mentioned gonadal tumours, including knockout mice for inhibin-α, anti-Mullerian hormone (AMH), and its type 2 receptor, namely AMHR2 (Matzuk et al. 1992, 1995, Behringer et al. 1994, Mishina et al. 1996). The inhibin-α mutant mice developed gonadal tumours affecting principally Sertoli cells, whereas the gene disruption of either Amh or Amhr2 in mouse resulted in Leydig cell hyperplasia, indicating an essential effect of AMH pathway on the control of Leydig cell proliferation. Moreover, the double inhibin-α/Amh-deficient mutant mice gave rise to the earlier development of granulosa/Sertoli cell tumours and multifocal Leydig cell neoplasia (Matzuk et al. 1995). Since previous works have demonstrated the physical and functional interactions between menin protein, encoded by the MEN1 gene, and the effectors of TGF-β/BMP pathway, Smad1, 3 and 5, in various cell types and tissues (Kaji et al. 2001, Sowa et al. 2003), we decided to investigate whether there is a common molecular basis among LCTs developed in heterozygous Men1 mice and those found in mice with deficiency in AMH pathway. Here, we report that the deregulation of AMH/BMP and TGF-β pathways is evidenced in the LCTs developed in heterozygous Men1 mice, accompanied by the altered expression of its target genes. Our data thus provide insights into the molecular basis of the physiopathological consequence of Men1 inactivation in Leydig cells.

**Materials and methods**

**Men1 mutant mice and genotyping**

Mice carrying an inactivated Men1 allele (Men1<sup>+/<i>T</i></sup>) were generated as described previously (Bertolino et al. 2003b). All animal experiments were conducted in accordance with accepted standards of human animal care and were approved by the International Agency for Research on Cancer’s Animal Care and Use Committee. PCR analyses were performed to determine the presence of the wild-type and targeted Men1 alleles as described previously (Bertolino et al. 2003b).

**Cell lines and transfection assays**

MA10 (mouse Leydig cell line) cells were cultured in Waymouth’s MB 752/1 medium (Invitrogen Inc.) supplemented with 20 mM HEPES, 15% (v/v) horse serum (Invitrogen) and 25 μg/ml gentamycin at 37 °C with 5% CO<sub>2</sub>. Mouse Leydig tumour cell (MLTC) cells were cultured in RPMI 1640 medium (Sigma–Aldrich Corp.) supplemented with 10% fetal calf serum. Mouse embryonic fibroblasts were grown in DMEM containing 25 mM glucose and supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 100 μM β-mercaptoethanol (Sigma), and LCT10 cells were grown in the same medium at 37 °C with 5%
CO₂, except supplemented with 15% (v/v) horse serum, 2.5% (v/v) fetal bovine serum (Sigma) and without β-mercaptoethanol. Chinese hamster ovary (CHO) cells were grown in standard DMEM. Cells were transfected using lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions.

Plasmids

The expression vectors containing MEN1 cDNA inserted in pCI-neo vector (Promega) either in sense, referred to as pCI-M1S, or in opposite direction, as pCI-M1AS were used for menin reconstitution experiments (Wautot et al. 2000). Furthermore, two MEN1 mutations pCI-1384delAGG and pCI-Arg415Ster were also generated (Hussein et al. 2007). XVent2-luciferase plasmid has been kindly provided by Dr K W Cho (University of California at Irvine, USA). The construct containing Amhr2 cDNA inserted in pCDM8 vector (Gouedard et al. 2000) was used for positive control of AMHR2 detection. The whole length MEN1 cDNA was cloned in pGex-4T1 (Amersham Biosciences) for glutathione-S-transferase (GST) pulldown assay. The construct was verified by DNA sequencing.

Protein extraction, immunoprecipitation, GST pulldown and immunoblotting analysis

Enriched nuclear and cytoplasmic protein fractions from cells were prepared and analysed by western blotting as described previously (Wautot et al. 2000). The following used primary antibodies were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA, USA): anti-menin (C19, 1:7500), anti-Smad1 (1:1000), anti-Smad4 (1:1000) and anti-Smad5 (1:750) antibodies. Anti-phospho-Smad1 polyclonal antibody (1:1000), anti-Smad4 (1:1000), anti-Smad3 (1:3000), anti-p27 (1:1000), anti-pSmad1 (1:1000), anti-Cdk4 (1:1000, Santa-Cruz Biotechnology) antibodies against menin (C19, 1:5000), anti-actin monoclonal antibody from ICN (1:50 000, Aurora, CA, USA), HRP-secondary antibodies from Amersham. Anti-AMHR2 antibody was used as positive control of AMHR2 detection. The whole length MEN1 cDNA was cloned in pGex-4T1 (Amersham Biosciences) for glutathione-S-transferase (GST) pulldown assay. The construct was verified by DNA sequencing.

RT-PCR and northern blot analysis

For the detection of Amhr2, total RNAs were extracted from cells or testis, with single-step RNA extraction system (TRI-REAGENT, Sigma). cDNA was synthesised with Superscript II reverse transcriptase (Invitrogen). The expression of Amhr2 and 3β-hydroxy steroid dehydrogenase and actin transcripts was studied by RT-PCR using primers and conditions described previously (Hussein et al. 2007). For northern blot analysis, the standard conditions (Racine et al. 1998) were used to detect Amhr2 transcript with a probe generated from the above-described RT-PCR. For the detection of Smad transcripts, purified Leydig cells were prepared and checked from Swiss male CD-1 mice at 8 weeks of age as previously described (Racine et al. 1998), and total RNAs were extracted from purified Leydig cells and subjected to RT-PCR analysis using primers shown on Supplementary Table 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/. Briefly, reverse transcription was performed in a total of 20 μl with the First-Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics) using 1 μg RNA, AMV reverse transcriptase and random primers p(dN)₆ as recommended by the manufacturer. PCR was carried out in 25 μl PCR buffer containing 3 mM MgCl₂, with 2 μl appropriate cDNA, 400 nM forward- and reverse-specific primers, 2 mM of each dNTP and 0.5 U Taq polymerase. The PCR protocol used an initial denaturation step at 95 °C for 3 min followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and elongation at 72 °C for 45 s. Amplified products were analysed by electrophoresis on a 10% polyacrylamide gel containing tris-borate-EDTA (TBE) buffer and stained with ethidium bromide.

Histopathological and immunohistochemical analyses

Testes were collected from wild-type and heterozygous Men1 mutant mice and fixed in 4% buffered formalin for at least 24 h, followed by dehydration and paraffin embedding. Histopathological analysis was carried out on 3 μm sections stained with haematoxylin–eosin. Immunohistochemical staining was performed as described previously (Bertolino et al. 2003a), using antibodies against menin (C19, 1:500), anti-p18 (1:3000), anti-p27 (1:1000), anti-pSmad1 (1:1000), anti-Smad1 (1:1000), anti-Smad3 (1:1000), anti-Smad4 (1:1000), anti-Smad5 polyclonal (1:1000) and anti-Cdk4 (1:1000, Santa–Cruz Biotechnology) antibodies. In all these analyses, the control without primary antibody was systematically included to rule
out the non-specific staining due to secondary antibodies (data not shown).

Dual-luciferase reporter assay

For the reporter assay, 1 μg/well of XVent2 plasmid, together with a co-reporter vector expressing Renilla luciferase driving by a thymidine kinase promoter (pRL-TK, Promega) at a ratio of 1:100 (10 ng) were transfected in triplicate in LCT10 cells using six-well plates by lipofectamine 2000. Menin’s effect on XVent2 reporter was assessed by the transfection of 1 μg/well of the vectors expressing either wild-type or mutant menin. Cells were incubated at 37 °C and harvested 48 h after transfection. Cell lysates were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) in a luminometer tube (Dynatech Laboratories Inc., Chantilly, VA, USA) according to manufacturer’s instructions. Luciferase activity was measured and normalised for transfection efficiency with the activity of a co-transfected thymidine kinase promoter/Renilla luciferase reporter.

Results

Reduced AMHR2 expression in Men1 Leydig cell tumours

To investigate whether there is a deregulation of AMH pathway in Leydig cell lesions developed in male heterozygous Men1 mutant mice, we have firstly analysed the expression of Amhr2 transcript using both semi-quantitative RT-PCR (Fig. 1A) and northern blot (Fig. 1B) in the testes from male heterozygous Men1 mutant mice at 20 months of age. Surprisingly, we found that, although Amhr2 is known to express specifically in normal adult Leydig cells, its expression was downregulated in the testes from all tested male heterozygous Men1 mutant mice, invaded by a massive amount of Leydig cells. To know whether the expression of the Amhr2 gene is reduced at the protein level, we have checked its expression by western blot analysis. The results demonstrated that AMHR2 was clearly reduced in the testes from tested male heterozygous Men1 mutant mice at 20 months of age (Fig. 1C), compared with those from the age-matched control mice, especially considering that the tumours are mainly composed of Leydig cells.

Decreased expression and activities of Smads in mouse Men1 Leydig cell lesions

We have further analysed the expression of the receptor-regulated Smads of AMH/BMP pathway, namely Smad1 and 5, in Leydig cell lesions from Men1 mutant mice. To make sure that Smad genes are expressed in normal mouse Leydig cells as previously reported (Jiao et al. 2002, Hu et al. 2003), we have carried out the detection of their transcripts by RT-PCR analysis using purified Leydig cells. Our data showed that all the tested Smad genes are expressed in the latter (Supplementary Fig. 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/), as well as in two different mouse Leydig tumour cell lines MA10 and MLTC. To detect eventual alteration of Smad expression in Leydig cell lesions developed in Men1 mutant mice, we have used immunostaining analysis as it allows comparing more specifically their expression in normal Leydig cells with that in Leydig cells lesions. To this end, immunostainings using both anti-Smad1 and anti-phosphorylated Smad1 antibodies were performed on the testes respectively from male
heterozygous Men1 mutant mice at 12 months \((n=5)\) and 20 months of age \((n=5)\), and the age-matched control mice \((n=5\) for each age group). The results showed that Smad1 protein could be readily detected by immunohistochemistry using anti-Smad1 antibody in the cytoplasm and nucleus of Leydig cells, and also in the seminiferous epithelium from the control mice (Fig. 2A), similar to what has previously been reported (Jiao et al. 2002, Hu et al. 2003). On the contrary, its expression was detectable only in the cytoplasm, but not in the nucleus in Leydig cell hyperplastic lesions (Fig. 2B). Smad1 expression was further reduced in the cytoplasm and undetectable in the nucleus in Leydig cells tumours (Fig. 2C). The similar result has been obtained when using an antibody recognising phosphorylated Smad1, the activated form of Smad1 with mainly nuclear localisation (Fig. 2D–F). The results were confirmed by western blot analysis of nuclear-enriched fractions of protein extracts using anti-phosphorylated Smad1 antibody, as we found the expression of phosphorylated Smad1 greatly reduced in the testes from the mutant mice, compared with those from the control mice (Fig. 2J).

Immunostaining of Smad5 demonstrated that, in contrast to what observed in the testes from Men1 wild-type mice \((n=5,\) Fig. 2G), its expression was virtually absent in both Leydig cell hyperplastic lesions and tumours from Men1 mutant mice \((n=5\) for each group, Fig. 2H and I). The results were confirmed by western blot analysis of nuclear-enriched fractions of protein extracts using anti-Smad5 antibody. As illustrated in Fig. 2K, nuclear Smad5 could be detected in none of the five mice tested except one (TTU4) where a trace of nuclear Smad5 expression was found.

Smad3, a menin-interacting protein like Smad1 and 5, is the receptor-regulated Smad of TGF-β pathway.

Figure 2 Reduced Smad1 and 5 expression in mouse Men1 Leydig cell lesions. Immunohistochemical analysis with anti-Smad1 (A–C), anti-phosphorylated Smad1 (D–F) and anti-Smad5 (G–I) antibodies were performed on testes sections from wild-type mice at 20 months of age (A, D and G), from Men1\(^{+/-}\) mice at 12 months of age (B, E and H) and from Men1\(^{-/-}\) mice at 20 months of age (C, F and I). Insets show an amplified view of testis sections. The scale bars are 50 \(\mu m\). (J and K) Western blot analysis of phosphorylated Smad1 and Smad5 expression. Nuclear protein extracts (20 \(\mu g\)) from two testes isolated from wild-type Men1 mice (T1WT and T2WT) and testes from six Men1\(^{-/-}\) mice at about 20 months of age (TTU1, TTU2, TTU3, TTU4, TTU5 and TTU6) were immunoblotted, and revealed respectively with anti-phospho-Smad1 and anti-Smad5 antibodies. Nuclear extracts from MA10 cells treated with AMH were used as positive control. Protein loading was monitored by an anti-actin antibody. This is representative of three independent experiments.
Since this pathway is involved in the development of gonadal sex-cord stromal tumours in inhibin-α mutant mice, we investigated the expression and the activity of Smad3 in Leydig cell lesions in heterozygous Men1 mutant mice. Immunohistochemistry analysis showed that Smad3 expression was found in the cytoplasm and nucleus of normal seminiferous epithelia and Leydig cells \( (n=3; \text{Fig. 3A}) \), whereas it was undetectable in the nucleus in Leydig cell hyperplastic lesions \( (n=6) \) nor in Leydig cell tumours \( (n=4) \) from Men1 mutant mice \( (\text{Fig. 3B and C}) \).

Smad4 is needed for the activity of both BMP and TGF-β receptor-regulated Smads. In order to know whether Men1 inactivation can affect Smad4, we have examined its expression in Men1 Leydig cell lesions. Immunostaining of Smad4 showed that it is expressed in normal seminiferous epithelia and in Leydig cells both in the cytoplasm and nucleus \( (n=4; \text{Fig. 3D}) \). As shown respectively in Fig. 3E and F, this expression was markedly reduced, but not totally absent, in Leydig cell hyperplastic lesions \( (n=5) \) and Leydig cell tumours \( (n=5) \). Western blot analysis revealed a detectable but much weaker expression of Smad4 in the nuclear fraction of the testes from Men1 mutant mice, compared with the control mice \( (\text{Fig. 3G}) \), whereas Smad4 was undetectable in the cytoplasmic fraction, suggesting the decreased total expression of Smad4 in Leydig cell tumour.

Taken together, our data indicate a reduced expression and activity of Smad1, 3, 4 and 5 in mouse Men1 Leydig lesions. In particular, the reduced expression of Smads was more evident in the nucleus of Leydig cell lesions, indicating the reduction of active form of Smads.

**Menin interacts with Smad1 and regulates AMH/BMP target promoter in Leydig cells**

The above-revealed close correlation between abnormal expression and activities of the components of AMH/BMP pathway and menin inactivation in Leydig cells lesions led us to further analyse the physical interaction between menin and Smad1 in Leydig cells, reported previously in osteoblasts \( (\text{Sowa et al. 2003}) \). Our analysis showed that the endogenous menin was co-immunoprecipitated by an antibody against Smad1 with protein extracts prepared from mouse Leydig cell line, MA10 cells treated by BMP2 stimulation. Consistent with the previously reported work, Smad4 was detected in the same immunoprecipitated protein complex \( (\text{Fig. 4A}) \). We noticed that such an interaction has not been observed upon AMH stimulation in our condition \( (\text{Fig. 4A}) \). We have
Indeed, our data demonstrated that the incubation of nuclear extract from both BMP2- and AMH-stimulated MA10 cells with GST fusion protein containing full-length menin led to binding of endogenous Smad1 (Fig. 4B), although its presence is rather weak in upon AMH stimulation. To further study the functional interaction of menin and Smads, we have investigated the effect of menin on the transactivation of target genes by this pathway. To this end, we have carried reporter assay using a widely used AMH/BMP target promoter (Gouedard et al. 2000), XVent2, in a Men1-deficient Leydig cell line, LCT10, generated in our laboratory from a Leydig cell tumour developed in a male heterozygous Men1 mouse. LCT10 cells keep basic features of Leydig cells, but do not carry the wild-type allele of the Men1 gene (Hussein et al. 2007). Moreover, they have higher expression levels of AMH/BMP pathway components than those found in mouse Men1 Leydig cell tumours (data not shown), except Smad5, most likely due to the selection during their establishment. We found that menin re-expression in LTC10 cells enhanced significantly the transactivation of XVent2 promoter by more than onefold increase, whereas the expression of two mutants of menin, either with a truncation, pM1-Arg415ter, or with an in-frame deletion, pM1-1384delAGG, did not exert such an effect (Fig. 4C).

Figure 4 Menin interacts with Smad1 and regulates AMH/BMP target promoter XVent2-luciferase in Leydig cells. (A) Co-immunoprecipitation of endogenous menin and Smad1. Immunoprecipitation of 1 mg protein extracts from MA10 cells stimulated respectively by BMP2, AMH and not stimulated was performed using anti-Smad1 antibody. Western blot analysis of resulting immunoprecipitated proteins was done using anti-menin and anti-Smad4 antibodies. Input; 20 μg MA10 total protein extracts. Note that menin can be co-immunoprecipitated with Smad1 upon BMP2 stimulation, whereas Smad4 is co-immunoprecipitated upon AMH and BMP2 treatment. This is representative of several independent experiments. (B) GST pulldown assay was performed on MA10 cell lysates. GST (G) or GST-menin (GM) proteins immobilised on Sepharose beads were incubated with lysates of MA10 cells treated (+) or not (−) with AMH or BMP2. Co-sedimented Smad1 protein was detected with anti-Smad1 antibody. Coomassie Blue staining demonstrated that the same amount of GST and GST menin proteins was used. Input represents 5% of the total cellular lysate. (C) Effect of menin re-expression on the activity of XVent2 promoter. To measure the activity of the XVent2-luciferase reporter gene in LCT10 cells co-transfected with XVent2 promoter and vectors expressing either menin (pCI-M1), or menin mutants (pCI-1384delAGG and pCI-Arg415ter), or an empty vector (pCI-neo) and the vector with inverted Men1 cDNA insert (pCI-M1AS), were analysed using dual-luciferase reporter assay. All results are expressed as mean ± s.d. Cells were transfected with the indicated plasmids in triplicate of at least five independent experiments. *P<0.005.

Deregulation of AMH/BMP and TGF-β targets in mouse Men1 Leydig cell lesions

It is known that CDK inhibitors p18 and p27 are both regulated by AMH/BMP pathway and menin protein (Ha et al. 2000, Karnik et al. 2005, Milne et al. 2005). Moreover, the mice with p18 ablation developed Leydig cell tumours, while the double p18/p27 knockout mice accelerated the development of Leydig cells tumours with a shorter incubation period and higher penetrance (Franklin et al. 2000). Furthermore, we have recently shown that menin re-expression in Men1-deficient LCT10 cells increased the expression of both p18 and p27 (Hussein et al. 2007). To analyse their expression in mouse Men1 Leydig cell lesions, we performed immunohistochemical analysis using anti-p18 and p27 antibodies. The result showed that nuclear staining of active forms of p18 and p27 was readily detectable in a substantial proportion of Leydig cells in Men1 wild-type mice (Fig. 5A and D), whereas the same staining was greatly reduced in Leydig cell hyperplastic lesions (n=5; Fig. 5B and E) and totally disappeared in all the tested Leydig cell tumours (n=5; Fig. 5C and F). Our data demonstrate that there is an inactivation of p18 and p27 in mouse Men1 Leydig
cell tumours where the Men1 gene is completely inactivated.

We have also checked the expression of Cdk4, a known target gene of TGF-β pathway (Ewen et al. 1993, Serrano et al. 1993, Hirai et al. 1995). Immunohistochemical analysis demonstrated that Cdk4 was intensively expressed in both Men1 Leydig cell hyperplastic lesions and tumours (Fig. 5H and I), compared with what detected in the control mice (Fig. 5C), suggesting its deregulation. In addition, we have analyzed the expression of other factors involved in cell cycle progression, including cyclin A2, D2 and E. However, we did not find any obvious expression of these genes in Leydig cell lesions (data not shown).

**Discussion**

The current study demonstrate the deregulation of AMH/BMP and TGF-β pathways, from the decreased presence of its type 2 receptor AMHR2 to the reduced nuclear expression of the terminal effector Smad4, as well as the altered expression of several target genes in mouse Men1 Leydig cell lesions. Considering the very similar tumour phenotype observed in Amh and Amhr2 knockout mice and the known interaction between menin and several Smads, this finding revealed in the current study may represent the molecular basis implicated in the development of Leydig cell lesions in Men1 mutant mice. Our results afford thus specific clues to the understanding of the mechanisms of Leydig cell tumour development triggered by Men1 inactivation.

Indeed, the downregulation of Amhr2 in Leydig cell lesions is reminiscent of the molecular context existing in Amh and Amhr2 mutant mice. Since menin is also known to interact with the R-Smad proteins of TGF-β pathway and that Smad3 expression and its sub-cellular location were altered in mouse Men1 Leydig cell lesions, the more severe tumour phenotype observed in Men1 mutant mice, compared with that found in Amh or Amhr2 mutant mice, could result from the synergistic effects of the deregulated both AMH/BMP and TGF-β pathways due to menin inactivation. In fact, Matzuk et al. (1995) have shown that the double Amh and inhibin-α knockout mice did develop Leydig cell tumours, whereas the latter is only a rare event in Amh single mutant mice. Our data confirmed thus their results and highlighted further that the components of both AMH/BMP and TGF-β pathways are vital in the control of Leydig cell proliferation. On the contrary, the lack of Sertoli cell lesions in Men1 mutant mice may indicate that the inhibin-α pathway is not

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**Figure 5** Altered expression of AMH/TGF-β target genes in mouse Men1 Leydig cell lesions. Immunohistochemical analysis with respectively anti-p27 (A–C), anti-p18 (D–F) and anti-Cdk4 (G–I) antibodies. (A, D and G) Testes from Men1 wild-type mice at 20 months of age. (B, E and H) Testes from Men1+/− mutant mice at 12 months of age. (C, F and I) Testes from Men1−/− mutant mice at 20 months of age. Insets show an amplified view of testis sections. The scale bars are 50 μm. This is representative of three independent experiments.
completely affected in Men1 inactivation, albeit the reduced Smad3 expression, revealing a cell type-specific role of menin in TGF-β pathway in testes interstitial tissue.

The downregulation of Amhr2 observed in mouse Men1 Leydig cell tumours is rather surprising, as menin is so far only known to interact with the down-stream R-regulated Smads. We have failed to detect the direct regulation of menin on a 350 bp proximal Amhr2 promoter by reporter assay (data not shown). Consequently, the mechanisms leading to the downregulation of Amhr2 remain elusive. However, it is known that the downregulation of TGF-βR2 is widely observed in human tumours, considered as a part of multi-step mechanisms of tumorigenesis allowing tumour progression. Interestingly, Ratineau et al. (2004) have recently shown that knockdown of menin expression in a rat embryonic duodenal cell line, intestinal epithelial cell (IEC-17), led to the downregulated expression of TGF-βR2. The reduced expression of Amhr2 observed in this tumour model could result from the similar mechanism, and thus could be an important molecular event in Leydig cell lesions due to Men1 inactivation. The further work is needed to elucidate the occurrence of Amhr2 down-regulation in mouse Men1 Leydig cell lesions.

Several studies using cellular models have previously shown the physical and functional interaction between menin and Smad proteins, including Smad1, 3 and 5, playing an important role in the control of cell proliferation and hormone production in pituitary and parathyroid cells (Kaji et al. 2001, Lacerte et al. 2004, Sowa et al. 2004b), and in osteoblasts (Sowa et al. 2003, 2004a). Importantly, our data demonstrated that these functional and physical interactions also exist in Leydig cells, indicating that menin’s biological and onco-suppressive role in Leydig cells may imply a similar molecular mechanism than that observed in the above-mentioned endocrine cells. Interestingly, our data also revealed that the expression of target genes of AMH/BMP and TGF-β pathways, including that of p18, p27 and Cdk4, were deregulated, suggesting that the alteration of the components of these two pathways in Leydig cells may result in the dysfunction of their regulation on proteins involved in cell cycle control. We have recently showed that menin re-expression in Men1-deficient Leydig cells resulted in a cell cycle blockade from G1- to S-phase transition, and augmented the expression of p18 and p27 (Hussein et al. 2007). The downregulation of p18 and p27 in mouse Men1 Leydig cell lesions revealed in the current study further highlights their role in the control of Leydig cell proliferation and in tumorigenesis of Leydig cells triggered by Men1 inactivation. Cdk4 is one of the key factors that control the transition of cell cycle from G1- to S-phase, and has previously been shown to be a target of TGF-β pathway in Leydig cells (Cipriano et al. 2001). Furthermore, Rane et al. (1999) have reported that mice deficient for Cdk4 manifested a reduced Leydig cell population, while mice expressing activated Cdk4 developed Leydig cell hyperplasia. Recently, Ratineau et al. (2004) have also demonstrated that knockdown of menin expression resulted in a significant increase of Cdk4 expression in IEC-17 cells. The current study suggests that the intensive expression of Cdk4 in mouse Men1 Leydig cell lesions, together with the downregulation of p18 and p27 is a part of molecular consequences of Men1 inactivation. It would be important to clarify whether and how its altered expression is caused by menin inactivation.

AMH/BMP and TGF-β pathways are well known for their important biological role in Leydig cells. The deregulation of these pathways due to Men1 inactivation revealed by the current study should thus also shed light onto the probable biological and endocrine functions of menin in Leydig cells. Furthermore, considering that menin may act, as recently reported (Dreijerink et al. 2006), as a co-activator of nuclear receptor-mediated transcription and that Leydig cells are among androgen target cells, the study of menin’s role in Leydig cells could be of importance for understanding how the Men1 gene functions in cells where nuclear receptor-mediated transcription plays a crucial role in cell proliferation control.

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References

the MEN1 gene in familial multiple endocrine neoplasia type 1 and related states. *Human Molecular Genetics* 6 1169–1175.


Biondi CA, Gartside MG, Waring P, Loffler KA, Stark MS, Magnuson MA, Kay GF & Hayward NK 2004 Conditional inactivation of the MEN1 gene leads to pancreatic and pituitary tumorigenesis but does not affect normal development of these tissues. *Molecular and Cellular Biology* 24 3125–3131.


Sowa H, Kaji H, Canaff L, Hendy GN, Tsukamoto T, Yamaguchi T, Miyazono K, Sugimoto T & Chihara K 2003 Inactivation of menin, the product of the multiple endocrine neoplasia type 1 gene, inhibits the commitment of multipotent mesenchymal stem cells into the osteoblast lineage. Journal of Biological Chemistry 278 21058–21069.


