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Reconstituted expression of menin in *Men1*-deficient mouse Leydig tumour cells induces cell cycle arrest and apoptosis

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ABSTRACT

Multiple endocrine neoplasia type 1 (MEN1) is a hereditary syndrome caused by the inactivation of the responsible gene, *MEN1*. To date, the lack of *MEN1*-deficient cell lines derived directly from MEN1 tumours has hampered the detailed study of the *MEN1* gene. We have established several stable *Men1*-deficient Leydig cell tumour (LCT) lines derived from a Leydig cell tumour developed in a male heterozygous *Men1* mutant mouse. Our data show that these cell lines maintain the basic characteristics of Leydig cells in terms of both androgen synthesis and gene expression. Interestingly, reconstituted menin expression in one of *Men1*-deficient LCT cell lines resulted in cell growth inhibition, suggesting that the function of cell growth suppression of the menin pathway, apart from menin itself, is essentially preserved in these cells. Furthermore, we show that menin re-expression in these *Men1*-deficient cells leads to a block in the transition from G0/G1 to S phase of the cell cycle and an increase in apoptosis, accompanied by a marked increase of $p18^{INK4C}$ and $p27^{Kip1}$ expression. The current study therefore highlights the importance of menin expression in cell cycle and cell survival control in endocrine cells, and may provide insights into the mechanisms of tumour suppression by menin in related endocrine tumours.

1. Introduction

Multiple endocrine neoplasia type 1 (MEN1, OMIM 131100) is an autosomal dominant inherited disorder characterised by the occurrence of multiple hyperplasia and tumours of the parathyroids, endocrine pancreas, anterior pituitary, and adrenal cortex. In addition, other endocrine or non endocrine tumours can also be seen in MEN1 patients, such as carcinoid tumours, thyroid follicular adenomas, lipomas, angiofibromas and collagenomas.[1] The responsible gene for the syndrome, *MEN1*, is considered as a tumour suppressor, since its complete inactivation can often be evidenced by LOH (loss of heterozygosity) analysis in MEN1 tumours.[2,3]

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The *MEN1* gene encodes a 610 amino acid protein named menin,[4] which is mainly localised in the nucleus. Menin interacts with a number of proteins, incuding JunD,[5] several members of nuclear factor-κB,[6] Smad1, 3 and 5,[7,8] nm23,[9] RPA2,[10] FancD2,[11] ASK,[12] MLL1 and MLL2.[13–15] The fact that the majority of its protein partners are transcription factors and co-factors allows to hypothesise that menin could be involved in transcriptional regulation. Indeed, it has recently been reported that this protein may regulate the expression of several genes, including telomerase,[16] several hormones,[17,18] and the cyclin-dependant kinase inhibitors involved in cell cycle control, such as $p18^{INK4c}$ and $p27^{Kip1}$.[14,19,20] However, the *in vivo* relevance of the interactions between menin and its partners is so far poorly understood. The biological function of menin thus remains to be elucidated.

One of the difficulties in studying *MEN1* is the lack of *MEN1*-deficient cell lines derived directly from MEN1 tumours. This hampered greatly the detailed study of cellular and molecular effects of *Men1* gene expression. Nevertheless ectopic expression of menin represses proliferation and tumourigenesis of Ras-transformed NIH3T3 cells,[21] even though it is difficult to pinpoint the molecular mechanisms involved using such a cellular model, due to the presence of substantial levels of endogenous menin. Reduction of menin expression by transfected antisense cDNA in the rat duodenal crypt-like cell line, IEC-17, increased cell proliferation.[22] In addition, menin is critical for TGF-β induced inhibition of cell proliferation in pituitary tumour-derived cells.[8] Interestingly, Schnepp and colleagues have demonstrated that the reconstituted menin expression in *Men1*-deficient immortalised mouse embryonic fibroblasts (MEF) restored the sensitivity to apoptosis in response to UV and TNFα.[23] These findings suggest that menin regulates cell proliferation. However, it remains to demonstrate whether and how the cell proliferation inhibition function of menin exists in endocrine cells and whether it is disturbed in MEN1 tumours.

More recently, mouse knockout models for the *Men1* gene have been generated by several laboratories,[24–28] including ours. Interestingly, in addition to the endocrine tumours corresponding to those commonly described in MEN1 patients, we have observed the development of Leydig cell tumours with high frequency in our heterozygous *Men1* mice.[24] In order to carry out mechanistic studies on tumourigenesis related to *Men1* gene inactivation, we have attempted to establish stable *Men1*-deficient endocrine tumour lines derived from the tumours developed in heterozygous *Men1* mice. Here we report the successful establishment of several stable Leydig cell tumour (LCT) lines derived from a Leydig cell tumour developed in a male heterozygous *Men1* mutant mouse. All the established LCT lines have lost the wild-type *Men1* allele, and thus are the first *Men1*-deficient endocrine tumour lines so far reported. Most importantly, our data demonstrate that the reconstitution of menin expression exerts inhibitory effects on cell growth in one of these cell lines, through the mechanisms involving cell cycle blockage and apoptosis, as well as the increased expression of the inhibitory factors of cell cycle.

2. Materials and methods

2.1. Tumour isolation and primary culture

Testis tumours were excised from transgenic mice. The tumour capsule was gently removed, and the tumour cells were mechanically dissociated and pressed through a 40-μm Cell Strainer (Becton Dickinson Labware, Franklin Lakes, NJ). After one wash, they were plated in 12-well plates at about 10^6 cells per well, in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 25 mM glucose and supplemented with 15% (v/v) horse serum (Invitrogen), 2.5% (v/v) foetal bovine serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin and 2 mM L-glutamine, and incubated in humidified air with 5% CO_2 at 37 °C. When cells reached about 50% confluency, they were transferred to 100-mm plates (Falcon) by trypsinisation with 0.05% (w/v) trypsin/0.5 mM EDTA. After about 3–5 passages, cells entered senescence and the medium was changed twice a week until cells restarted to grow (about 2 months in average). Then cells were divided twice a week and were cloned by limited dilution (one cell per well in 96-well), with the cloned lines being referred to as Leydig cell tumour (LCT) lines.

2.2. Cell lines and transfection assays

MA-10 (mouse Leydig cell line) cells were cultured in Waymouth's MB 752/1 medium (Life Technologies, Inc.) supplemented with 20 mM HEPES (GIBCO), 15% (v/v) horse serum, and 25 μg/ml gentamycin (GIBCO) at 37 °C with 5% CO_2. Mouse embryonic fibroblast (MEF) were grown in DMEM containing 25 mM glucose and supplemented with 10% (v/v) foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 100 μM β-mercaptoethanol (Sigma) at 37 °C with 5% CO_2. Cells were transfected using lipofectamine 2000 (Invitrogen; Cergy Pontoise, France) according to the manufacturer's instructions.

2.3. Cytogenetic analyses

Metaphase spreads of cultured cells were prepared and stained by 4′,6′-diamidino-2-phenylindole (DAPI) according to the protocol previously described.[29]

2.4. Men1 mutant mice and genotyping

Mice carrying an inactivated *Men1* allele ($Men1^{+/T}$) were generated using a targeting vector, as described previously.[25] 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. Southern blot analyses were performed to determine the presence of the wild type and targeted *Men1* alleles, by hybridising *Bam*H1 and *Bgl*II digested genomic DNA with a *Men1* gene probe, located immediately upstream of exon1.[25] The wild-type allele produced a 5.6 kb fragment, the targeted allele a 3.6 kb fragment. Genotyping by PCR was performed as described previously.[24]

2.5. RT-PCR

Total RNAs were extracted from cells or testis, with single step RNA extraction system (TRI-REAGENT, Sigma, l'Ile d'Abeau, France). cDNA was synthesised with Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France) and the amount of cDNAs were adjusted by dilution to produce equal amounts of hypoxanthine guanine phosphoribosyl transferase (HPRT) amplicon. The expression of AMHRII (anti-mullerian hormone receptor type II), P450 cholesterol side-chaine cleavage (P450scc), 3β-HSD (3β hydroxy steroid dehydrogenese), P450 17α-hydroxylase/C17-20 lyase (P450c17), LHR (luteinising hormone receptor), FSH receptor (follicle stimulating hormone receptor), steroidogenic acute regulatory protein (StAR), was studied by using primers and conditions previously described.[30] For the Southern blot hybridisation of RT-PCR products, 20 μl of the reaction products for the LCT lines and 4 μl for MA10 and wild type mouse testis were blotted, hybridised with specific probes as previously described.[30]

2.6. Plasmids

For the generation of an expression vector containing *MEN1* cDNA, pCI-NtM1 containing a tagged human *MEN1* cDNA,[31] was deprived of the tag using PCR with the following primers: Men1-FW: GCTTGTCGACGGATCCACGCGTTTGCCATG; Men1-RV: GTAGGAGCGGCTGAGGCTG. The *MEN1* cDNA without tag thus generated was inserted in pCI-neo vector (Promega) either in sense, referred to as pCI-M1S, or in opposite direction, as pCI-M1AS. Two *MEN1* mutations were separately introduced into pCI-M1S construct (pCI-1384delAGG and pCI-Arg415ter). The pEGFP-C1 vector (clontech) was used to amplify the EGFP gene (Enhanced Green Fluorescent Protein) by PCR using two primers containing *lox P* sites flanking the amplified gene. The PCR fragment thus obtained, named *loxP*-EGFP-*loxP*, was cloned in pCI-neo vector (Promega) after digestion with *NheI*/*MluI*. *MEN1* cDNA obtained from pCI-M1S was inserted in the resulting plasmid (pCI-*loxP*-EGFP-*loxP*) in the downstream sequence of *loxP*-EGFP-*loxP* to obtain the pCI-*loxP*-EGFP-*loxP*-M1 construct (pLEM).

2.7. Protein extracts preparation and immunoblotting

To prepare total protein extracts, cells were lysed at 4 °C in the buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% (v/v) Nonidet P-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptine, pestatin, 1 mM Na_3VO_4, 1 mM NaF, 1 mM β-glycerophosphate. The lysates were clarified by centrifugation. Enriched nuclear and cytoplasmic protein fractions from Leydig tumour and cells were prepared and analysed by western blotting as described previously.[31] The anti-menin (C19, H300) polyclonal antibodies were purchased from Santa-Cruz Biotechnology (1:7500, California, USA). Anti-actin monoclonal antibody from ICN (1:50,000, Aurora, USA), anti-p18 polyclonal antibody, anti-p27 monoclonal antibody, anti-p21 polyclonal antibody, anti-cyclin D1 monoclonal antibody, and anti-cyclin E polyclonal antibody from Santa-Cruz Biotechnology (1:1000, California, USA), HRP-secondary antibodies from Amersham (Orsay, France). Protein bands were scanned and analysed by the Quantity One (BioRad).

2.8. Histopathological and immunohistochemical analyses

Tissues were collected from 34 to 85-week-old wild type and heterozygous *Men1* mutant mice and fixed in 4% (w/v) 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 (H&E). Immunohistochemical staining was performed essentially on serial sections, as described previously,[24] using antibodies against menin C19, 1:500.

2.9. Growth suppression of Leydig cells

Leydig cells were cultured at 50% confluence on 6 cm diameter dishes and transfected with 6 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen). A duplicate set of dishes was used for each transfection. After 48 h, cells were trypsinised and plated at 1×10^5 cells/well in 6-well plates in the medium containing G418 at 2.5 mg/ml to select the transfectants. After 15 days, the cells were Giemsa-stained and the number of foci was counted. For growth suppression study using inducible menin expression, cells were treated with 1 μM 4-hydroxytamoxifen (OHT) during 2 days. OHT-containing medium was replaced by normal medium. Cells were then cultured for 10 days, and scored for the number of foci formation.

2.10. Measurement of progesterone production

In order to have equal cell number for each cell line on day 2, cells were plated on day 0 in duplicate in 6-well plates in 2 ml of media, with LCT8, LCT9 and LCT10 at 3.5×10^5/well, LCT11 at 5×10^5 /well, MA10 at 5×10^5/well and MEF at 3.5×10^5/well. On day 2, the cells were washed with serum-free medium and placed in 2 ml of warm assay medium (DMEM/Nutrient Mixture F-12 Ham, supplemented with 1 μg/ml insulin, 10 μg/ml vitamin E and 5 μg/ml transferrine). Cells were respectively incubated with 50 ng/ml hCG (human chorionic gonadotropin) (Organon), 50 μM Forskolin (Sigma), 1 mM 8Br-cAMP (8-Bromoadenosine 3′:5′-cyclic Monophosphate, Sigma) and 1 μM 22(R)-hydroxycholesterol (Sigma) for 24 h at 37 °C and 5% CO_2. Culture medium was collected from treated and untreated cells and assayed by radioimmunoassays (RIA) in triplicate for total accumulated progesterone and testosterone as previously described.[32–34] Testosterone and progesterone polyclonal antibodies were kindly given by Maguelone Forest (Lyon, France).

2.11. Fluorescence-activated cell sorting analysis

On day 0, cells were seeded at a density of 3×10^5 cells/6 well plates. On day 1, cells were left untransfected (Mock) or transfected with empty vector (pCI-neo, Promega), pCI-M1S, or pCI-Arg415ter. 48 h later, cells were harvested, and 10^6 cells per sample were fixed in 4% (w/v) paraformaldehyde for 10 min at 4 °C. Cell pellets were washed in phosphate buffered saline,

pelleted, and incubated with anti menin antibody (Rabbit BL342, BETHYL) in PBS 1X containing 0.5% (w/v) saponin (Sigma)/3% (v/v) SVF / 1% RNasin for 45 min followed by washing with PBS 1X containing 0.5% (w/v) saponin/3% (v/v) SVF and pelleting. Next, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) antibody was added for 30 min. After another washing and pelleting, cells were resuspended in PBS 1X buffer containing SVF 3% and 3 μg/ml propidium iodide (PI; Molecular probes) and incubated for 5 min at 37 °C. Samples were analysed on a FacsCalibur (Becton Dickinson).

3. Results

3.1. *Establishment of Men1-deficient Leydig cell tumour lines*

The heterozygous *Men1* mutant mice develop endocrine tumours similar to those commonly observed in MEN1 patients. In addition, these mice presented sex-cord stromal cell tumours with high frequency.[24] In particular, Leydig cell tumours found in the testes from the male mutant mice

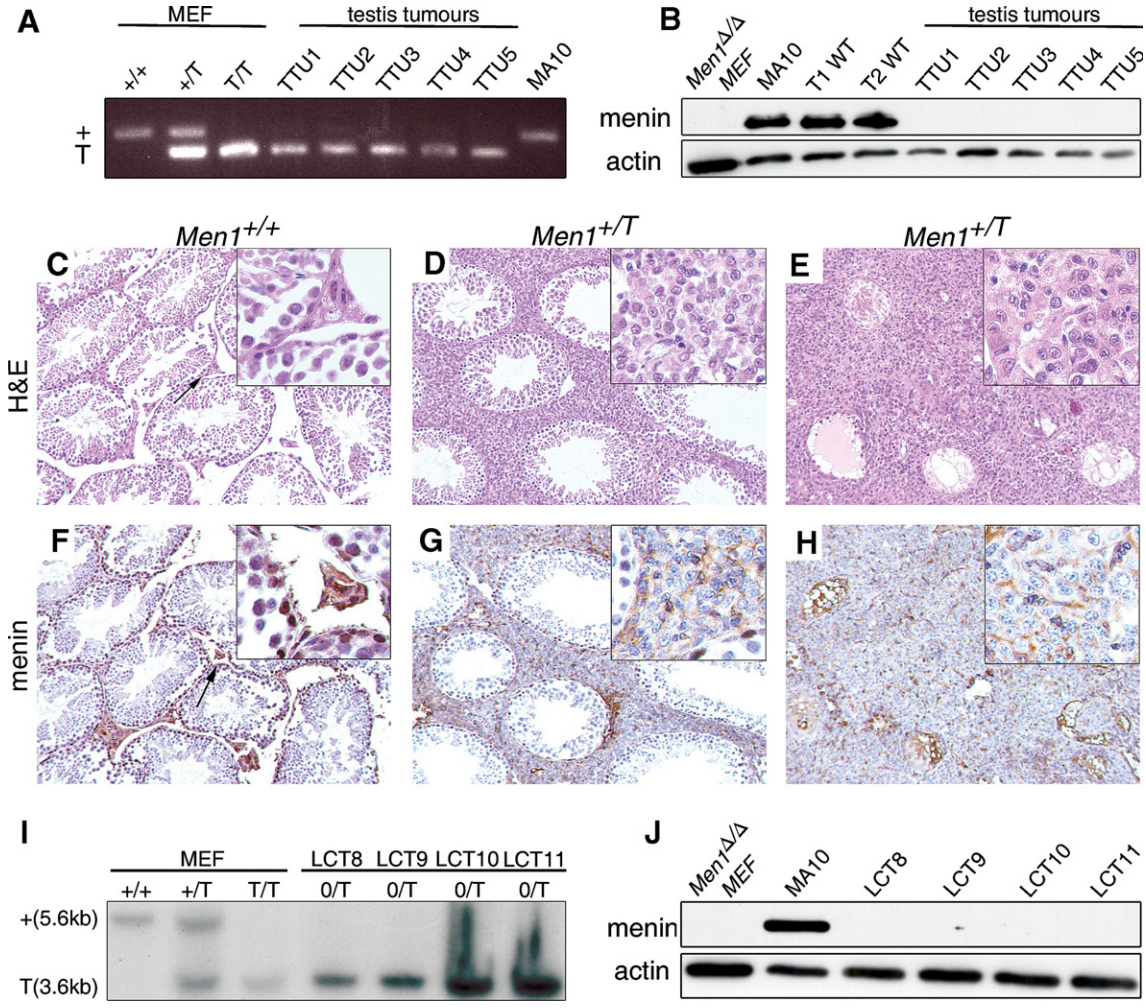

Fig. 1 – LOH analysis, *Men1* gene expression in Leydig cell tumours and derived lines. (A) PCR analysis was performed to check LOH. DNA samples extracted from MEF cells containing respectively wild-type (+/+), targeted (T/T) and heterozygous *Men1* allele (+/T) and from testis tumours (TTU1–TTU5) were genotyped with PCR. Note that the wild-type *Men1* allele is lost in all five testis tumours compared with control cells. (B) Western blot analysis of menin expression in Leydig cell tumours. 20 μg of nuclear protein extracts from *Men1*$^{\Delta/\Delta}$ MEF cells containing the deleted *Men1* allele,[25] MA10, two testes isolated from wild-type *Men1* mice (T1WT and T2WT) and Leydig cell tumours from five *Men1*$^{+/T}$ mice at about 20 months of age (TTU1, TTU2, TTU3, TTU4 and TTU5) were separated by SDS/PAGE, immunoblotted, and revealed with an anti-menin antibody. The protein loading was monitored by actin. Histological (C, D and E) and immunohistochemical analysis with an anti-menin antibody (F, G and H) performed on testes sections from wild-type mice (C and F) and from *Men1*$^{+/T}$ (D, E, G and H). Note that the presence of menin expression in normal Leydig cells (arrows in F), and the absence of menin expression in Leydig cell hyperplasia (G) and tumour lesions (H). Original magnification: ×10 (insets ×40). (I) Southern blot analysis of established LCT lines. The *Men1* wild-type allele (+) produced a 5.6 kb fragment, and the targeted allele (T) a 3.6 kb fragment after digestion of genomic DNA with *Bam*HI and *Bgl*II, and hybridisation with a *Men1* probe.[25] The genotype of each cell line is indicated on the top of the gel. Note that the wild-type *Men1* allele is lost in all four LCT lines compared with control cells. (J) Western blot analysis of menin expression in LCT lines, MA10 and *Men1*$^{\Delta/\Delta}$ MEF cells. The protein loading was monitored by actin.

behaved aggressively and often developed metastases. The LOH analysis carried out previously showed that the wild type allele was systematically lost in Leydig cell tumours, suggesting the complete inactivation of the *Men1* gene. To confirm this result, LOH analysis was carried out with Leydig cell tumour samples from five *Men1*$^{+/T}$ mice at about 20 months of age in the current study. The result showed that the loss of the wild type allele was detected in all the tested samples (Fig. 1A). In parallel, to verify the absence of menin in the Leydig cell tumours, we performed western blot analysis on the proteins prepared from the same five tumours. The absence of menin expression was found in all the tested Leydig cell tumours (Fig. 1B). Furthermore, we have carried out immunostaining using anti-menin antibody on testis samples from 20 month-old wild type mice, or from *Men1* heterozygous mice with hyperplastic (10 months old) or tumour (20 months old) lesions. Five mice were tested for each group. In contrast to the mainly nuclear staining observed in Leydig cells from wild-type mice (Fig. 1F), menin expression was absent in the hyperplastic and tumour lesions from *Men1*$^{+/T}$ mice (Fig. 1G and H). These results demonstrate that there is a complete inactivation of the *Men1* gene in the Leydig cell tumours.

We carried out the primary culture of Leydig cell tumours isolated from 20 month-old *Men1*$^{+/T}$ mice carrying one wild

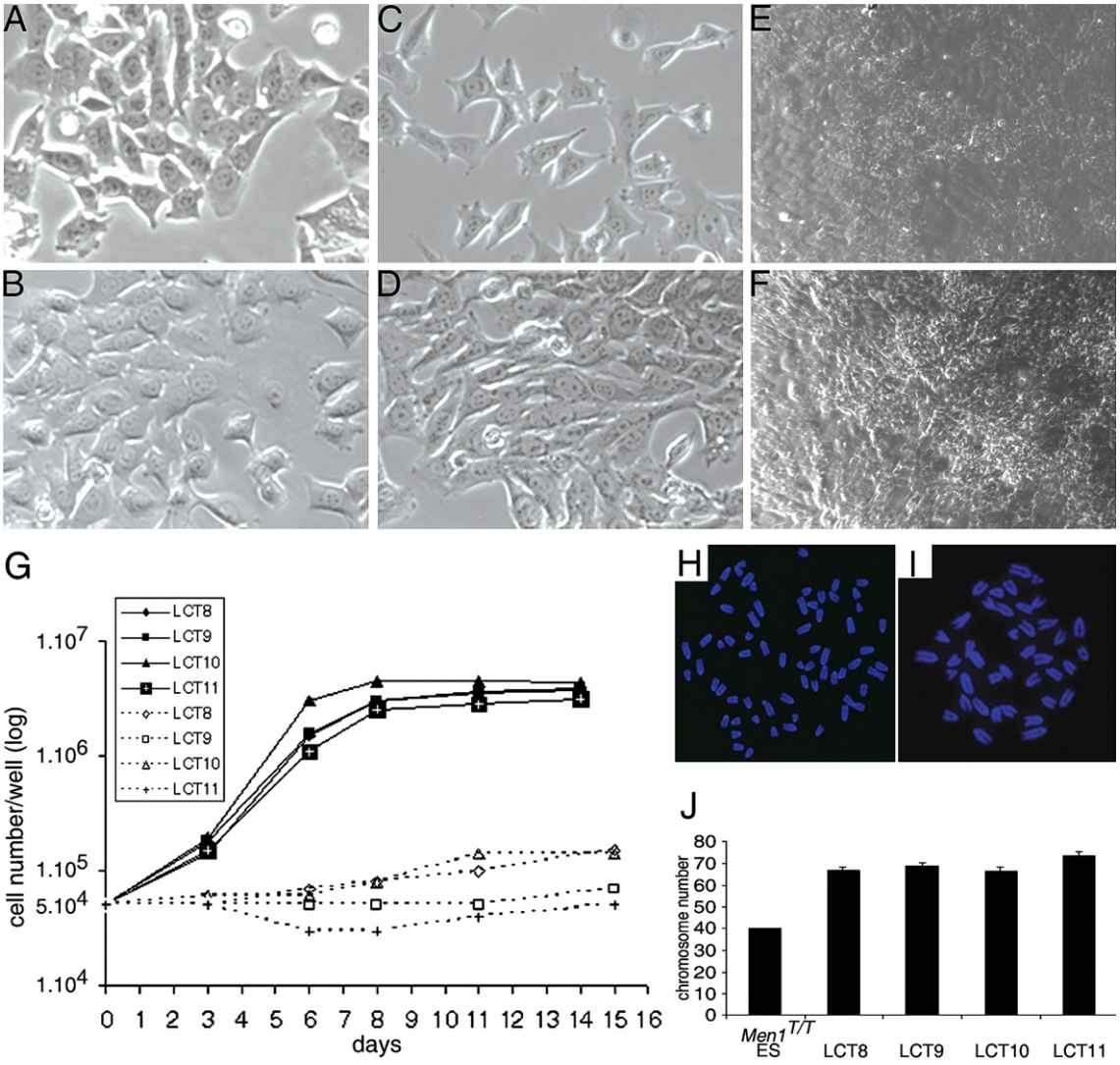

Fig. 2 – Morphology and cell growth of LCT lines. Microscopic view of LCT8 (A), LCT9 (B), LCT10 (C) and LCT11(D) at thirtieth passage in culture (Phase contrast, original magnification ×20). Microscopic view of LCT10 (E) and LCT11 (F) forming ultimately multilayered foci after 2–3weeks of culture (phase contrast, original magnification ×6). (G), Growth curves of LCT lines cultured in routinely used medium (continuous line) or in the medium containing 1% horse serum (dot line). Cells were plated at 35 mm dishes and the cell number was counted at the indicated times. In each case, a representative result from one of the two independent experiments is shown. Metaphase chromosomes were prepared from Leydig cell lines, and representative metaphase spreads stained with 4′,6′-diamidino-2-phenylindole (DAPI) from LCT10 and *Men1*$^{T/T}$ ES cells are respectively shown in (H) and (I). (J), the number of chromosomes scored for LCT lines shows great increase compared with that of *Men1*$^{T/T}$ ES cells.

type *Men1* allele and one targeted allele containing a NeoTK cassette inserted in intron 2,[25] and obtained cells starting to continually grow after a period of senescence (see Section 2). By limited dilution cloning of one mass culture established from a Leydig cell tumour, we obtained four stable clones, namely LCT8, LCT9, LCT10 and LCT11, which have so far been cultured more than 30 passages in our laboratory without visible change in morphology and growth behaviour. Southern blot analysis showed that the loss of the wild-type *Men1* allele occurred in all these four LCT lines (Fig. 1I), consistent with the LOH analysis previously carried out in Leydig cell tumours from $Men1^{+/T}$ mice.[24] Western blot analysis failed to detect menin expression with total protein extracts prepared from the above four lines, confirming thus that the targeted allele of the *Men1* gene is non-functional (Fig. 1J).

3.2. *Characteristics of Men1-deficient LCT lines in culture*

Morphologically, the LCT cells have a rounded shape at low densities (data not shown) and become relatively flat at 50–70% confluence (Fig. 2A–D), displaying an abundant cytoplasm with many protrusions, and large and light nucleus with three or more prominent nucleoli, a sign of intense cellular activity.

After plating at about 30% confluence, LCT lines began to grow exponentially after a lag period of 2 to 3 days. LCT10, and both LCT8 and LCT9 proliferated respectively with an average doubling time of 18 h and 24 h, while LCT11 grew more slowly and had a doubling time of about 30 h. After growing to complete confluence at day 7, all the LCT lines slowed down their growth, but did not stop completely (Fig. 2G). When maintained in culture for a longer period (2–3 weeks), LCT cell lines ultimately formed multilayered foci, as shown in Fig. 2E and F. The results indicate that these LCT cells may have lost, at least partially, contact inhibition. We also noted that all the LCT lines cultured in a low concentration of horse serum (1%) nearly stopped growing, indicating that they were largely serum dependent (Fig. 2G).

Chromosome spreading analysis showed that these cells were aneuploid having 66 ± 2 chromosomes per cell for LCT8, LCT9 and LCT10 on average, and 73 chromosomes for LCT11, indicating chromosome instability in these cells (Fig. 2H and J).

3.3. *Expression of Leydig cell markers in Men1-deficient LCT lines*

To confirm the cell type origin and determine the expression profile of the genes involved in Leydig cell function in the above LCT cells, the transcription of a panel of selected genes was analysed by RT-PCR (Fig. 3). The Sertoli-cell specific follicle-stimulating hormone (FSH) receptor was totally absent in all the *Men1*-deficient LCT lines, ruling out a Sertoli cell origin for these lines. On the contrary, with the exception of 3β-HSD expression in LCT8, the genes known to be involved in testicular steroidogenesis in Leydig cells were detected in all of the LCT lines, including StAR, a mitochondrial phosphoprotein involved in the translocation of cholesterol across the mitochondrial membrane,[35,36] P450scc, a mitochondrial enzyme,

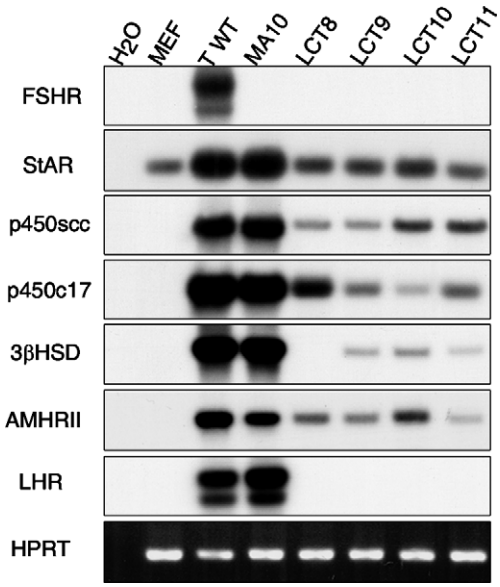

Fig. 3 – Transcriptional expression of various steroidogenic genes in LCT lines. Southern blot hybridisation of RT-PCR products of FSHR, StAR, P450scc, P450c17, 3βHSD, AMHRII and LHR. The amount of RNA was monitored by HPRT. Results shown are representative of three independent experiments.

and P450c17, which resides in the smooth endoplasmic reticulum, both belonging to the cytochrome P450 superfamily,[37] and 3β-HSD expressed mainly in the gonads and adrenals.[38] No LH receptor transcripts were detected in *Men1*-deficient LCT lines, whereas AMH type II receptor, known to be specifically expressed in Leydig cells and crucial to the initiation of AMH signal transduction,[39] was detected in all the LCT lines. We noticed that the expression of the majority of the above Leydig cell markers was significantly weaker in *Men1*-deficient LCT lines compared with the positive controls, MA10 cells and testis from wild-type mice, as we could detect the expression of the markers (AMHRII, 3βHSD and p450scc) by northern blot in MA10 cells, but failed in LCT lines (data not shown). We noticed that StAR expression was repeatedly found in MEF cells, but totally absent in other tested cells lines, such as Hela and HBL100 cells (data not shown). The presence of StAR in MEF cells may be related to its embryonic origin.[36]

3.4. *Steroid production in Men1-deficient LCT lines*

To determine the steroidogenic potential of *Men1*-deficient LCT lines, we assessed their ability to produce progesterone following the addition of hCG, Forskolin, 8Br-cAMP, and 22R-hydroxycholesterol, which stimulate steroidogenesis at different levels of the signalling cascade.[40,41] We noted that LCT lines produce varying levels of progesterone in response to these stimuli, apart from LCT8 line which had the ability to produce progesterone only in response to 22R-hydroxycholesterol stimulus (Fig. 4A). However, these levels were low compared with those in MA10 cells which have higher basal progesterone production (Fig. 4A and B). Nevertheless, in rel-

ative terms, the progesterone production by these cell lines in response to Forskolin, 8Br-cAMP and 22R-hydroxycholesterol, reached a level comparable to, or even higher than that found in MA10 cells (Fig. 4C–F). None of the LCT lines produced progesterone in response to hCG treatment, likely due to the lack of LH receptor (Fig. 4A and C). Testosterone production was not detectable in any of the LCT lines, as was the case in MA10 (data not shown).

3.5. *Suppression of cell growth by reconstituted menin expression*

As the *Men1* gene is completely inactivated in LCT cells, it should be interesting to investigate whether the cell growth of *Men1*-deficient LCT lines can be inhibited by reconstituted menin expression. To this end, LCT9 and LCT10 lines were subjected to transfection with pCI-M1S containing wild-type *MEN1* cDNA, followed by G418 selection and colony formation assay. We found that LCT10 cells transfected with pCI-M1S formed consistently reduced numbers of foci (Fig. 5D), about 2.5 fold, (Fig. 5G), compared with cells transfected with an empty vector (pCI-neo, Fig. 5B). No reduction in the number of foci was observed when LCT10 was transfected with an inverted *MEN1* cDNA construct pCI-M1AS (Fig. 5C), or with two mutants of *MEN1* cDNA (pCI-*1384delAGG* and pCI-*Arg415-ter*), as shown in Fig. 5E and F. Western blot analysis showed that both wild type menin and mutant menin were ectopically expressed (Fig. 5H). Although *MEN1* mutant constructs

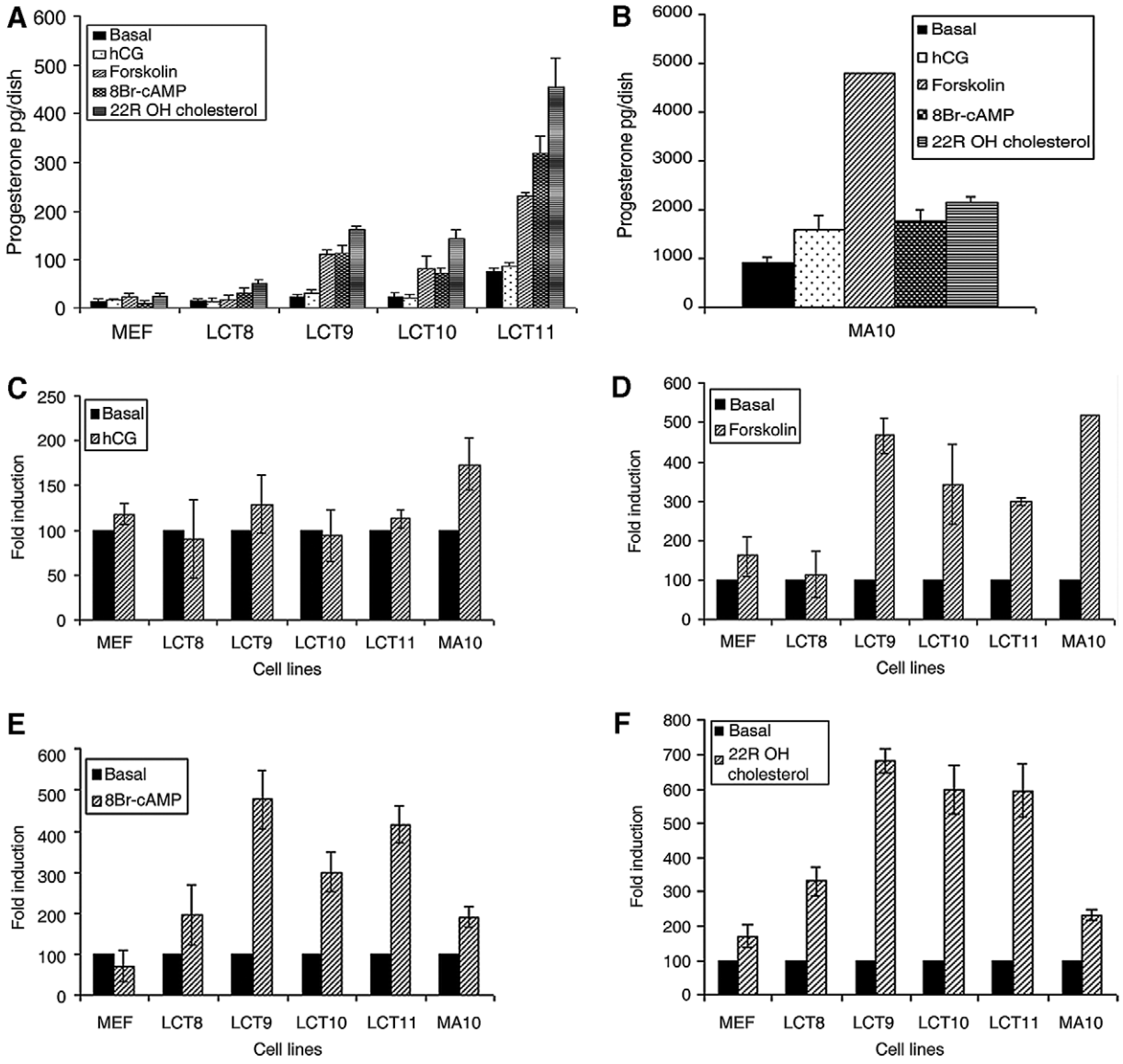

Fig. 4 – Steroidogenesis in LCT lines. Cells were treated or not with 50 ng/ml of hCG, 50 μM forskolin, 1 mM 8-Br-cAMP and 1 μM of 22R-hydroxycholesterol, and subjected to progesterone detection by RIA. *Men1*$^{+/+}$ MEF and MA10 cells were used respectively as negative and positive control in all experiments. The data correspond to the mean of triplicate determinations ±standard variation of steroid production from duplicate cultures for each treatment group, either shown as pg/well (A and B), or as fold change (C–F). All the experiments reported have been repeated at least twice with independent cell preparations, with only a representative result being shown.

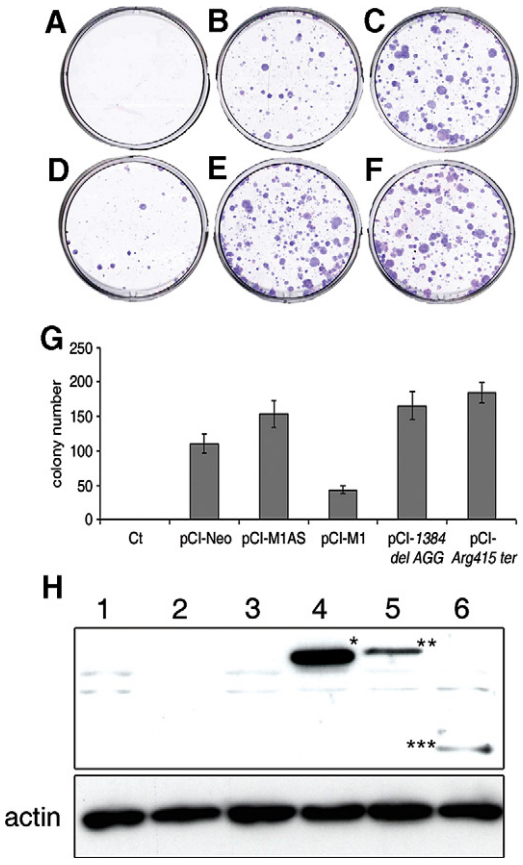

Fig. 5 – Inhibition of cell proliferation by reconstituted menin expression in LCT10 cells. LCT10 line was either untransfected (A), or tranfected respectively with the empty vector pCI-Neo (B), inverted *MEN1* cDNA construct pCI-M1AS (C), pCI-M1S (D), two mutants of *MEN1* cDNA, namely pCI-1384 *del AGG* (E) and pCI-*Arg415ter* (F). After 15 days of G418 selection, Giemsa-stained colonies were photographed and the number of colonies was scored (G). The results represent the mean of two independent experiments ± standard variation. (H) Western blot analysis of menin expression in LCT10 line transfected with different constructs. 20 μg of cell lysates were immunoblotted with anti-menin antibody. Lane 1, untransfected cells; Lane 2, cells transfected with the empty vector; Lane 3, transfected with pCI-M1AS; Lane 4, transfected with pCI-M1; Lane 5–6, transfected respectively with pCI-1384 *del AGG* and pCI-*Arg415ter*. * corresponds to wild type menin, ** and * correspond respectively to the two mutant menin, 1384 *del AGG* and *Arg415ter*. The protein loading was monitored by actin.**

expressed less than the wild type menin, due probably to the enforced degradation of the mutated *MEN1* gene,[42] there was no effect of cell growth inhibition observed, even proportional to their expression level. The result indicates that the reconstitution of menin expression is sufficient to inhibit cell growth in LCT10 cell line. The same effect has not been observed in LCT9 line (data not shown).

To confirm the above result, the effect of reconstituted menin expression on cellular proliferation in LCT10 line was further studied using an inducible expression system. To this end, pLEM, a construct containing *MEN1* cDNA preceded by a stop-sequence flanked by *loxP* sites was firstly introduced into LCT10 cells. The transfectants, called LEM1 cells, were then stably transfected with the pCre-ER construct expressing a tamoxifen-inducible Cre recombinase (Fig. 6A). The ability of double transfectants thus obtained, named CER2, to re-express menin under the induction of tamoxifen, was confirmed by both immunohistochemistry analysis (Fig. 6B) and western blot (Fig. 6C). To study the consequences of reconstituted menin expression on cellular proliferation of LCT10 cells, the double transfectants were treated with tamoxifen for 2 days, and then cultured in normal medium for 10 days for foci formation assay. The result showed that the number of foci formed was significantly reduced in double transfectants treated by tamoxifen, 3-fold less than that obtained in untreated cells (Figs. 6D and E). The same treatment did not result in any significant change in control cells (LEM1) which do not contain Cre recombinase (Fig. 6E). The data confirmed that cell proliferation of LCT10 cells can be inhibited by menin re-expression.

3.6. *Reconstituted menin expression results in cell cycle blockage and apoptosis*

To gain insights into cellular and molecular mechanisms of the cell proliferation inhibition by reconstituted menin expression, we determined the steady-state distribution of cell cycle phases in the cells with or without menin re-expression by flow cytometry analysis (Fig. 7A). As shown in Fig. 7B, 69, 5, and 14% of menin-reconstituted cells are in G0/G1, S, and G2/M respectively compared to 55, 12, and 28% of control cells. Consistent with this, cells transfected with an empty vector or mutant menin showed a similar cell cycle distribution to that in mock cells. These results demonstrate that menin re-expression increases the number of cells in G_1 but decreases that in S and G_2/M, indicating a block in the transition from G0/G_1 to S phase. Cell cycle arrest is often accompanied by an increase in apoptosis. We therefore sought to determine whether menin re-expressing cells alter the basal level of apoptosis compared with menin-null cells in our culture conditions. Figs. 7A and C shows that cells re-expressing menin contain 10% subdiploid cells (apoptotic cells), which is more than double than that found in menin negative cells. Taken together, these data indicate that menin re-expression can trigger both cell cycle blockage at the transition from G0/G1 phase to S and apoptosis in *Men1*-deficient LCT10 cells.

3.7. *Altered expression of cell cycle control proteins upon menin re-expression*

As G0/G1 to S transition was found to be blocked in *Men1*-deficient LCT10 cells with menin re-expression, we analysed the expression of the factors involved in cell cycle control in these cells. As shown in Fig. 8, the expression of both $p18^{INK4c}$ and $p27^{Kip1}$ was markedly increased and that of p21 was slightly increased in pCI-*MEN1* transfected cells compared with pCI-neo transfected cells. Surprisingly, the expression of cyclin D1 was also increased, whereas that of cyclin E was not altered. The data are consistent with the observed G0/G1 cell

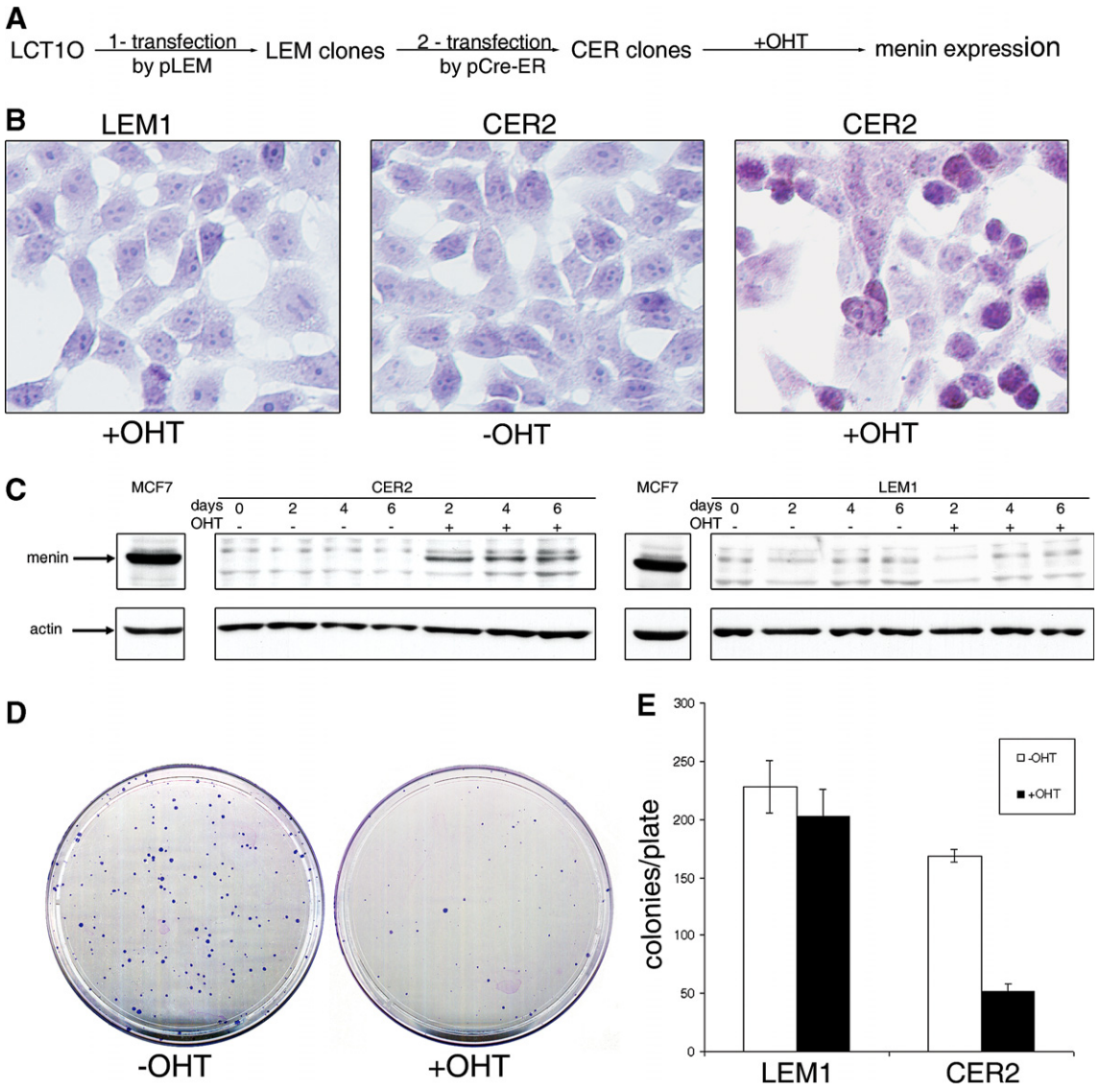

Fig. 6 – Inducible menin reconstitution in *Men1* deficient LCT10 cells. A, schematic representation of the inducible menin reconstitution in *Men1*-deficient LCT10 cells. B immunohistochemical analysis with an anti-menin antibody of control cells (LEM1) treated with OHT, and double transfectants, CER2, treated or not with OHT. Original magnification: ×40. C, menin protein levels analysed by Western blotting. Note that we could not detect menin protein in control cells (LEM1) treated or not with OHT during 6-day and in non-OHT-treated CER2 cells, whereas the menin protein levels in CER2 cells are induced starting from 2 days after treatment. MCF7 was used as a positive control for menin detection, and protein loading was monitored by actin. D, colony formation assay of LEM1 and CER2 cells. 10^3 cells were plated onto 10-cm dishes, incubated with or without OHT for 10 days and colonies were scored following Giemsa staining. Representative plates are shown. E, Quantification of colony numbers of LEM1 and CER2 clones with or without OHT treatment. Means ± SD are shown for triplicate samples from three independent experiments.

cycle arrest with menin re-expression and suggest that $p18^{INK4c}$, $p27^{Kip1}$ and cyclin *D*1 are effectors of menin expression in these cells.

4. Discussion

Leydig cell tumours are rare testis interstitial tumours which are so far not considered being related to MEN1 pathology, whereas one case has been reported in a male MEN1 patient.[43] Although the clinical significance of the finding of this type of tumour in heterozygous *Men1* mutant mice remains elusive, the recent finding of the interaction between menin and nuclear hormone receptor[44] has raised further concerns about menin's biological role in ER or AR target cells, including Leydig cells. It would thus be interesting to study molecular mechanisms of the development of Leydig cell tumours in *Men1* mutant mice. We believe that LCT lines generated in the current work should provide useful tools for such an investigation. Our results on the marker expression and steroids production in these LCT lines afford several criteria establish-

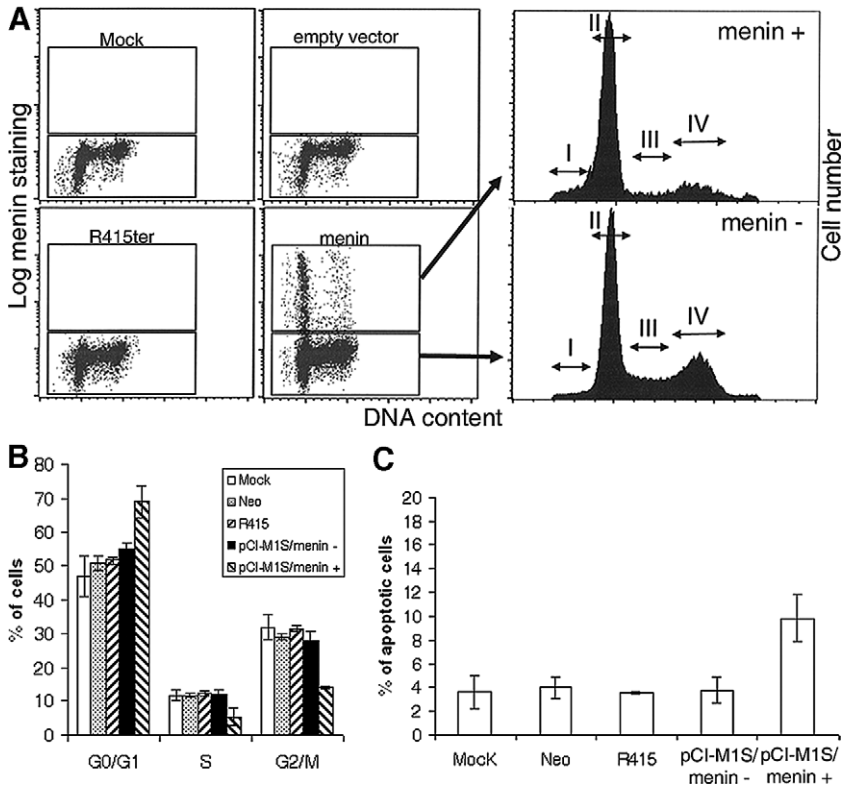

Fig. 7 – Analysis of cell cycle and apoptosis in *Men1*-deficient cells after menin reconstitution. A, cell cycle profiles of menin-deficient LCT10 (Mock), cells transfected with empty vector or pCI-*Arg415ter*, or cells transfected with pCI-M1S and electronically gated for menin negative (menin –) or menin positive (menin +) cells. Cells were seeded at a density of 3×10^5 cells/6-well plates and transfected with different constructs and allowed to grow for 48 h. Cells were then processed and stained with anti-menin antibody and propidium iodide as described in 'Materials and methods'. 'I' indicates subdiploid cells (apoptotic cells); 'II', cells in G0/G1, 'III', cells in S-phase; and 'IV', cells in G2/M. B, Percentage of cells in indicated phases of the cell cycle 48 h after transfection as determined by flow cytometry. C, percentage of apoptotic cells 48 h after transfection as determined by flow cytometry. Data shown were obtained from three independent experiments.

ing their origin of Leydig cells: (i) expression of Leydig cell specific markers and genes involved in steroid synthesis; (ii) lack of Sertoli cell marker FSH receptor; (iii) positive response to the stimulation for progesterone production. Interestingly, the response of LCT lines to the stimulation of steroid production in fold change was the same as or even higher than that found in MA10 cells, indicating that a substantial part of the machinery for steroid production, as well as its regulation, is still preserved in these cells. Thus, the established *Men1*-deficient LCT lines can also be used to study the regulation of steroid production and, more importantly, the eventual role of the *Men1* gene in this process and in the biology of Leydig cells.

Our analysis of LCT cells shows that these cells acquired numerous chromosome abnormalities, indicating there could be substantial chromosome instability occurring in these cells. These chromosome abnormalities could be the molecular basis of different cell behaviour that we observed among LCT lines, in terms of cell growth, endocrine function and sensitivity to menin reconstitution. We were not able to determine whether such chromosome instability existed in initial tumour cells, as the latter went into senescence soon after they were isolated from tumours. Intriguingly, both the study carried out in Drosophila model,[45] and those using fibroblasts and lymphocytes from MEN1 patients,[46] suggest that the inactivation of the *Men1* gene may result in genomic instability, whereas the CGH-array analysis of insulinomas developed in *Men1* β-cell specific mutant mice showed neither chromosomic nor micro-satellite instability.[47] It would be useful to improve primary tumour culture conditions to further address this issue.

The loss of the wild type *Men1* allele found in these cells is consistent with our previous and current detection of LOH of the *Men1* locus, further confirming that the *Men1* gene is completely inactivated in these tumours, as was the case in other endocrine tumours. It suggests thus *Men1* inactivation participates in tumourigenesis of Leydig cells in this mouse model. Since there is so far no reported successful establishment of stable cell lines from *Men1* tumours, the mouse LCT lines reported here represent the first endocrine cell lines generated directly from the tumours where the *Men1* gene is completely inactivated. It is particularly interesting that the cell growth of LCT10 line is sensitive to the reconstituted menin expression, further demonstrating the causal role of *Men1* inactivation in the tumourigenesis of mouse Leydig cells. Although LCT10 cells may accumulate many other genetic alterations, the *Men1* pathway seems to be essentially preserved. It is thus likely that other genetic changes may not affect the

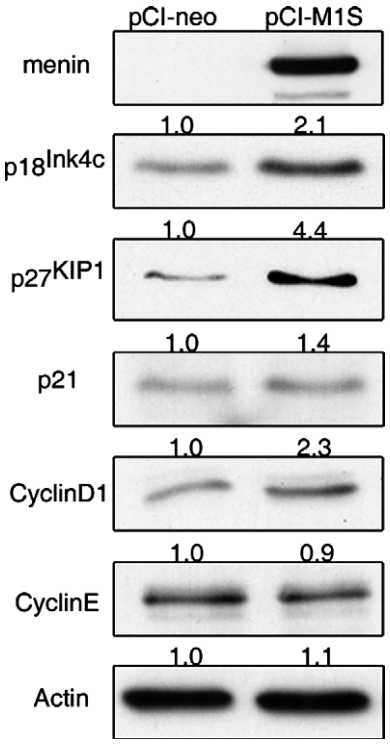

Fig. 8 – Altered expression of cell cycle regulators upon menin reconstitution. Western blot comparing expression of the indicated proteins in cells transfected with empty vector or pCI-M1. Actin serves as a loading control. This is representative of two independent experiments.

machinery necessary for menin's control on cell proliferation. This is reminiscent of other tumour cell models where the re-expression of deficient tumour suppressor genes, such as *BRCA1* and *NF2*, showed substantial effects on cell growth.[48,49] This cell line can thus be of importance in identifying the cellular and molecular effects of reconstituted menin expression by different approaches, which may shed light onto the mechanisms of cell proliferation controlled by the *MEN1* pathway. However, as Leydig cell tumours are not considered to be associated with MEN1 pathology, one should keep caution when extrapolating the results obtained from these cells to more common MEN1 tumours.

Indeed, by cell cycle distribution analysis, we have demonstrated that menin re-expression increased the number of cells in G1 phase, with a concomitant decrease in S and G2/M phases in *Men1*-deficient LCT10 cells, indicating a G1 arrest. Furthermore, our analysis also showed that this cell cycle arrest is accompanied by an increase in apoptosis. This is reminiscent of cell cycle arrest and apoptosis observed in *Men1*-null mouse embryonic fibroblasts (MEF) cells re-expressing menin previously reported by La and colleagues, even though it was reported that the effect of menin expression in these cells affects more likely at G2/M transition.[50] The difference observed could be explained by the difference in cell type or developmental stage. During the preparation of the current manuscript, Schnepp and colleagues has reported that the re-expression of menin in *Men1*-null MEF cells triggered a G1 arrest.[51] Our data thus not only confirmed menin's effect on cell cycle regulation, but demonstrated for the first time that such an effect can also occur in *Men1*-related endocrine tumour cells, providing the direct evidence showing that cell cycle disturbance and apoptosis could be the major factors in tumourigenesis of Leydig cells triggered by *Men1* inactivation. Interestingly, the simultaneous occurrence of G1 arrest and apoptosis has been observed in different circumstances, including the overexpression of cancer-related genes, such as p53, and the abnormal cellular process, including DNA damage.[52] It is believed that their occurrence may help to limit the growth of deregulated cells. It is therefore conceivable that menin expression may participate in such a cell protection to restrict the overgrowth of abnormal cells.

Menin has previously been shown to be involved in the regulation of different factors implicated in cell cycle control, including $p18^{INK4c}$ and $p27^{Kip1}$.[14,19] Indeed, the expression of these factors was increased in LCT10 cells with reconstituted menin expression. On the contrary, it is difficult to explain the increased expression of Cyclin D1 upon menin re-expression. However, the same molecular event has been described in a Ras-triggered G1 arrest in primary human epidermal cells.[53] Taken together, our work highlights the important role of menin expression in the control of both cell cycle and cell death.

Conflict of interest statement

None declared.

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